Handbook of Fungal Biotechnology

Second Edition, Revised and Expanded

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Preface

The fungal kingdom comprises one of the most diverse groups of living organisms. They are numerous, ubiquitous, and undertake many roles, both independently and in association with other organisms. The fungal species range from those the size of a few micrometers up to larger fungi with fruiting bodies ranging from several centimeters to meters, and in extreme cases they can develop into a colonial organism that covers many hectares. This diversity of form is also mirrored by functional diversity as fungi can virtually occupy all ecological niches, from slow-growing endolithic communities in the polar regions to highly specialized plant and animal pathogens, and rapid degraders of organic materials in tropical environments. The span of functional diversity in fungi makes them the richest model system in cell biology. Recent developments in molecular biology techniques, including DNA amplification, cloning and expression systems, and modern genomic and proteomic analyses have yielded the discovery of new compounds, and also offered tools to investigate, characterize, and exploit both new and long established fungal activities. Fungi have played a significant role in several biotechnology-based industrial processes and the formulation of a variety of compounds. Fungi are also the target of many biotechnological applications, from the development and production of noteworthy pharmaceuticals and industrial products to their use as systems for homologous and heterologous gene overexpression.

The bulk of available literature covers all the major aspects of general mycology, much of which is themed into broad subject areas such as systematics, ecology, biochemistry, pathology, and molecular biology. However, there is a scarcity of compiled literature related strictly to the basic principles of applied mycology and fungal biotechnology. Their broader implications in published literature are fragmented over several specialized journals. In order to attempt to bring together such a diverse field, I, along with coeditors, ventured to edit the five-volume series *Handbook of Applied Mycology* in 1992. This series offered a comprehensive treatment of basic principles, methods, and applications of mycology as an integrated and multidisciplinary subject. These five volumes presented and collated the major aspects of applied mycology and served as the standard reference for students, teachers, and researchers. Since 1992, significant developments in both biological sciences and industry have broadened the conceptual basis of fungal ecology, physiology, and biochemical processes that are directly relevant to biotechnological usage and manipulation. As a result it seemed timely to revise the original Volume 4 (*Fungal Biotechnology*) and to review the current developments and highlight advances in rapidly expanding areas of molecular technologies in industry, commercial production technology, and medical biotechnology.

The revised second edition of the *Handbook of Fungal Biotechnology* is intended to provide a broad and detailed introduction to the different aspects of fungal biotechnology, with chapters covering molecular technologies, commercial fungal applications, medical mycology, culture collections, legal aspects, and biosafety. The contributions include both reviews of existing fungal biotechnology applications and details of new processes that may become major applications in the future. For example, new chapters address topics ranging from cell biology of hyphae, protoplast fusion, metabolic regulation pathways, nuclei and chromosomes to genomics, gene clustering, gene cloning and sequencing, fungal mitochondrial genome, fungal genome and evolution, the role of GPF in fungal biotechnology, and DNA chips and

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microarrays. Coverage has been expanded on commercial applications of fungi, such as the application of genetic engineering for strain improvement, genetic importance of wine yeasts, fungi in brewing, alcohol production, fungal enzymes, role of chitin, polysaccharides, lipids in fungal biotechnology, production of citric acids, caretenoids, terpenoids, antibiotics, antifungal drugs, and antitumor and immunomodulatory compounds. Chapters that have direct or indirect significance in medical biotechnology have also been added.

The vast array of usage and properties is testimony to the countless ways in which mankind can harness the benefit of fungi; therefore, characterization techniques and methods of preservations of fungi, the recent development in biotechnology and intellectual property, access to genetic resources, and benefit sharing is essential. These challenging areas of fungal biotechnology are also covered in this book. Potential benefits and dangers of genetically modified foods and mycoherbicides are evaluated. Therefore, the aim of this handbook is to provide a snapshot in time as to the use of fungal biotechnology in different key areas, and to identify potential directions and possibilities for the future.

The subject areas related to agriculture, food, and environmental biotechnology are covered not in this volume but in the simultaneously published *Fungal Biotechnology in Agricultural, Food, and Environmental Applications* from the same editor.

In assembling this volume, I have collaborated with world-renowned scientists to illustrate many application areas of fungal biotechnology, from both industry and academia. The contributors are from a broad international background, and thus reflect the diverse activities occurring worldwide. I recognize serious difficulties in developing a comprehensive book on fungal biotechnology because of the range and complexity of the emerging knowledge. However, we have attempted to bring together pertinent information that may serve the needs of the reader, as a quick reference to a subject that might otherwise be difficult to locate, and by furnishing a starting point for further study. I hope that the comprehension of this material by readers will enhance their understanding and help them to gain new appreciation for many potential benefits of fungal biotechnology in a wide variety of fields. The book should be of great interest not only to students, teachers, and researchers but also to agricultural practitioners, mycologists, botanists, microbiologists, molecular biologists, food scientists, biochemists, ecologists, genetic engineers, environmental scientists, pharmacologists, and all those concerned with issues related to significant developments in the field of fungal biotechnology.

I am grateful to many colleagues for discussions and their advice during the preparation of this edition, and the academic niche of the Banaras Hindu University for the opportunity to complete this great task. I am grateful to many international authorities and specialists who have graciously consented to share their perspectives and expertise on the diverse applications of fungal biotechnology, and contributed chapters.

I am also indebted to Ms. Sandra Beberman, Vice President, Marcel Dekker, Inc., and Ms. Dana Bigelow, Production Editor, for their skill, patience, encouragement, guidance, and support.

Dilip K. Arora

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1

Cell Biology of Hyphae

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1 INTRODUCTION

Members of the fungal kingdom are present in almost every conceivable niche. Even though fungi are remarkably diverse, many fungi share common cellular characteristics that are instrumental to the success of fungal growth, development, proliferation, and survival. The purpose of this introductory chapter is to provide the reader with an overview of some of the fundamental aspects of one of the predominant forms of fungal structures—hyphae. The introductory chapter discusses the attributes that are common to many fungi as well as other organisms while also emphasizing some of the features that are unique to fungal species, as compared to other eukaryotes (some of the details will be discussed in depth in the following chapters of this book). Perhaps the primary recognizable difference between the hyphal "cell" and cells of other organisms is the predominantly coenocytic nature of the former. Characteristically, the hyphal cell harbors multiple nuclei that are evenly or unevenly distributed over relatively long distances of cytoplasmic continuity. Nonetheless, in this chapter, the term "cell" is used while discussing some of the fundamental as well as more unique attributes of hyphae. Identifying and understanding the nature of these attributes, and in particular, the regulatory mechanisms involved in orchestrating the growth of the fungal filament is an important step in the process of our intervention in fungal biology, be it curbing detriments or enhancing benefits these organisms are capable of exhibiting.

2 THE CELL WALL

The cell wall is a characteristic structure present in many organisms whose life style to grow in an environment with continuously changing water potential can be described as an absorptive one. Thus, the accumulation of large concentrations of osmotically active molecules requires the presence of a structure that will assist in maintaining the integrity of the cell membranes. In addition, the adventurous, and at times, invasive proliferation of the fungal filament in a variety of diverse environments requires the presence of effective mechanisms to defend the fungal cell from external perils. The cell wall is a prime example of an efficient mechanical exocellular defense system. Understanding the structure of the cell wall and the synthesis of its components is essential for elucidation of the processes involved in fungal growth and development. This understanding includes the fundamental aspects of filament elongation and branching as well as various aspects of differentiation, pathogenicity, absorption, and secretion.

2.1 Composition

The fungal cell wall accounts for approximately 25% of the mycelial dry weight. Approximately 80% of the cell wall dry weight is comprised of polysaccharides (Ruiz-Herrera 1992). The remaining 20% is comprised of proteins, lipids, and various inorganic salts. The predominant carbohydrate polymers found in different fungi are various forms of glucans and chitin. These sugars, synthesized and positioned in a nonuniform, yet highly regulated manner provide the external skeleton of the hyphal cell and are synthesized mainly at the apical region of the growing hyphae. Nonetheless, additional components (in particular—cell wall-associated proteins) are involved in determining the cell surface properties of the growing or nascent hypha. Cell wall associated proteins are involved in the restriction of cell permeability to detrimental compounds and/or proteins present in the environment. These cell wall proteins are also

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involved in recognition of other fungi and regulation of processes such as anastomosis, sexual and asexual partner recognition, and interactions (e.g., recognition and adherence) with various hosts (Lora et al. 1995; Saupe et al. 1996). The interactions between fungal cell wall proteins and potential hosts is predominantly based on protein–protein recognition, even though there are convincing records of carbohydrate–protein interactions with the carbohydrate supplied by either the fungus or the host (Cormack et al. 1999).

2.2 Synthesis

As the extension of the hyphal filament occurs mainly in the apical region, most of the biosynthetic machinery (and most likely some degradative machinery as well) involved in this process is trafficked to that region of the apical cell. As the main components of the hyphal cell are glucan and chitin, the glucan and chitin synthases must be positioned properly (in the vicinity of the extension or repair areas) and their activity regulated so as to produce the relevant polymer in a site-specific manner. The mechanism by which the polymers are extruded is yet unclear. The presence of several chitin synthases in filamentous fungi has been documented at the respective genomic databases (NCBI and other, specific, databases as mentioned in Section 7.3 of this chapter). Based on the available information, *Neurospora crassa* and *Aspergillus fumigatus* each have seven genes encoding for chitin synthases, whereas *Saccharomyces cerevisiae* has three. The differential expression and functional consequence of chitin synthase gene inactivation have demonstrated that different chitin synthases have different roles during fungal growth and development (for a detailed perspective see Chapter 30). Some chitin synthases are essential for maintaining proper hyphal rigidity and form and are required for hyphal elongation. The specific roles of other members of this gene family have yet to be elucidated. The cell wall biosynthetic (chitin and glucan synthases) as well as cell wall lytic (chitinases and glucanases) enzymes required for cell elongation and branching processes are conveyed to the required location, and at least in some cases, compartmentalized trafficking is carried out in membranous vesicles (Sietsma et al. 1996). The wall at the tip is plastic allowing the extension of the cell by insertion of new material. As extension progresses, the material at the former position of the apex is progressively rigidified as it becomes the lateral wall of the growing cell. This rigidification is brought about by the covalent cross-linking of wall materials, especially chitin and $\beta(1->3)$ glucans, and the hydrogen bonding of adjacent polysaccharide chains, especially chitin, to give microfibrils.

Many of the cell wall-associated proteins are heavily glycosylated. Many of the proteins secreted to the external face of the plasma membrane are linked via a remnant of the glycosylphosphatidylinositol anchor to the polysaccharide cell wall component. *N*-Glycosylation of proteins in the fungal endoplasmic reticulum is most likely very similar to that observed in mammalian cells, yet the process occurring in the Golgi apparatus is different than that described in mammals (Dean 1999). *O*-Glycosylation also occurs in filamentous fungi and is mediated by a conserved family of protein-mannosyl-transferases (Strahl-Bolsinger et al. 1999).

2.3 Hydrophobins

Hydrophobins are among the unique protein components of the fungal cell wall. These small proteins, secreted during a variety of developmental processes are present in most filamentous fungi and have, so far, not been found in nonfungal species (Talbot 1999). These proteins play essential roles in fungal adherence, development of aerial structures, and infection of host plants by phytopathogens (Ebbole 1997). Fungal hydrophobins are hydrophobic in nature and can be defined by the presence of eight cysteine residues that are spaced in a particular manner within the amino acid sequence. They have been divided into two classes, based on solubility characteristics brought about by differences in cysteine residue spacing and distribution of hydrophobic and hydrophilic amino acids within the hydrophobin polypeptide sequences (Wösten et al. 1999). Beyond the involvement of fungal hydrophobins in natural processes of fungal growth and proliferation, the unique attributes of these proteins have been the basis of several suggested industrial applications. These include the use of hydrophobins as molecular anchor points for industrial proteins/enzymes, by attaching them to hydrophobic plastic surfaces. Other potential uses include the production of a natural protein coating over artificial organs or other transplants (Kershaw and Talbot 1998).

Though many structural components and organizational features are common to a wide range of fungi, differences in cell walls can be observed among different fungal species. Furthermore, the composition and structure of the cell wall can diverge immensely during the growth and development of the fungal colony.

3 THE PLASMA MEMBRANE

The presence of a cell wall provides the fungal cell with the ability to survive and grow without the need to equalize the cellular osmotic potential to that of its environment. In fact, the difference in osmotic potential contributes to the ability of the hyphal cell to elongate and branch by creating turgor pressure. The primary active barrier between the fungal cell and the environment is the plasma membrane. As seen in other organisms, the plasma membrane is a selective divide involved in flow of material and information between the cell and its environment.

The maintenance of a plasma membrane potential and appropriate ion gradients are the basis of the ability to transport solutes in and out of the fungal cell. This ability is achieved via a variety of proton pumps, carrier proteins, and ion channels. *N. crassa* has been a prime model for analysis of

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proton pumping and membrane potential (Davis 2000). The maintenance of a pH gradient and a high membrane potential (≈ 200 mV, inside negative) is mainly dependent on the efflux of protons, rather than Na^+ (the common mechanism in animal cells). Nonetheless, even though the plasma membrane ATPase (a key element in the maintenance of the pH gradient) is essential, it is most likely that other processes contribute significantly to the stabilization of cytoplasmic pH. These, most likely, include the modulation of the passive membrane permeability to protons, modulation of oxidative metabolism (a source of cytoplasmic protons), modulation of various proton-coupled transporters, and modulation of the flux between the cytosol and storage organelles (the vacuole in particular). A comprehensive overview of the transport processes operating in the fungal plasma membrane and the tonoplast has been compiled by Garrill (1994).

Apart from the maintenance-related functions mentioned previously, the plasma membrane (and plasma membrane-associated proteins) plays a key role in additional biological functions of the hyphal cell. This includes serving as the anchoring structure for a variety of receptors involved in environmental sensing, a key location for initiation of endocytosis, and the final step in secretion. Furthermore, all steps of hyphal fusion, be it self-fusion or fusion occurring with other strains during vegetative and/or mating processes involve recognition and changes in the plasma membrane.

4 SEPTATION

Compartmentalization of the fungal cell is dependent under most circumstances on the formation of septa. The initiation of septum formation has been associated with the recruitment of actin to a specific site along the hyphal cell. The events that follow include the deposition of cell wall material outside the plasma membrane. The septal wall provides a structural component for maintaining the hyphal architecture, yet is perforated in a way that provides the maintenance of cytoplasmic continuity and organelle movement (including, in some cases, nuclei) between compartments. Septation results in the cessation of growth and nuclear division in subapical cells. These subapical cells provide a reservoir of growth potential for the extending hypha. In most fungi, the nuclei residing within the subapical compartments ultimately give rise to new branches and various spore types. Thus, septation is most likely involved in determining both cell size and shape.

In ascomycetes the septa generally appear to be homogeneous with a simple perforation while in basidiomycetes some septa are trilaminate. The primary septal plate carries at its center a thickened ring (the septal swelling) that surrounds the septal pore. Either face of the septal swelling is enclosed by the concave surface of a dome-shaped structure which is composed of a irregularly perforated multilayer membrane. This complex septal structure, termed the "dolipore septum" is characteristic of most basidiomycetes (excluding, for example, the rusts and the smuts). Occlusions within the pore and the parenthesomes on either side (in some fungi) delimit the pore domain (Moore 1985). The dolipore septum plays a role in nuclear migration, as the size of the pore and the parenthesome membrane provide a considerable barrier to nuclear movement. In fact, nuclear migration (as part of the dikaryon formation process) has been shown to be associated with disruption of the septum by lytic enzymes (Casselton and Economou 1985). Once the dikaryon is established, septal disruption no longer occurs. In contrast, it is important that septa remain intact for the maintenance of the binucleate stage. The cessation of septal disruption is probably one of the early postmating events regulated by the mating factors.

One of the major risks imposed by cytoplasmic continuity characteristic of many filamentous fungi is the catastrophic event of massive leakage. Such events may occur following mechanical or chemical damage to the cell wall, which in severe instances can result in impairment of the filament structure to the point where cell contents can no longer be retained. A key mechanism in the process of minimizing the damage following the formation of a rupture in the fungal cell is the induction of rapid cell wall repair activities (Ruiz-Herrera 1992) while at the same time activating a mechanism aimed at blocking the septal pores. One of the key factors involved in the latter process (in ascomycetes) is the Woronin body. Woronin bodies are proteinaceous granules that can reach a size of $\approx 0.5\,\mu$m. They are of peroxisomal origin (Jedd and Chua 2000; Keller et al. 1991) and usually reside along the peripheral regions of the fungal cell and in association with the septum. The Woronin body is comprised of a crystalline subunit made of a protein with a peroxisome-targeting signal that most likely indicates that these bodies originate from the peroxisomal membrane. Even though the Woronin bodies do not move along with normal cytoplasmic streaming, the dramatic increase in cytoplasmic flow following cell rupture may provide sufficient force to pry the Woronin bodies from their plasma membrane site of attachment, and provide the vehicle for a rapid direction of the bodies to the septal pore.

5 THE CYTOSKELETON

The hyphal cytoskeleton plays a key role in cell shape determination, maintenance, and growth. A broad spectrum of proteins interacts with the basic components of the hyphal cytoskeleton that is comprised of microtubules and actin filaments. The dynamics of the hyphal cytoskeleton that involves constant change and adjustment in concert with cellular changes is characteristic of filamentous fungi. Furthermore, it is most likely that cytoskeletal elements interact directly with components of the cell wall. Thus, structure, sensing, and cellular response are closely associated with the hyphal cytoskeleton.

In contrast to many other cells types (including yeasts and higher eukaryotic cells) the distances that some cellular

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components must travel within the elongated hyphal cell are enormous. The continuous supply of vesicles (produced in the Golgi apparatus) to the apical cluster is critical for tip growth. This cluster of vesicles also termed the Spitzenkörper (apical body) appears as a dense spherical body with no discrete outline and is associated only with growing hyphae (apical cells) or immediately prior to the formation of new branches. The vesicles within the apical body range in diameter from 30 to 400 nm. The content of these vesicles, which most likely includes (among other proteins) enzymes involved in cell wall biosynthesis, has yet to be properly determined. Perhaps the most studied components of the apical body are a subpopulation of the smaller vesicles—the chitosomes. The pioneering work of Braker et al. (1976) established the linkage between these vesicles and chitin synthase activity. Following the isolation of genes encoding chitin synthases and the availability of antichitin synthase antibodies, localization of chitin synthases to chitosomes by immunohystochemistry was made possible (Sietsma et al. 1996). The capacity to transfer cargo along the cytoskeletal backbone of the hyphal cell is provided by molecular motors that are responsible for both forward and retrograde transport along the hyphal filament. The proteins that provide the ATP-driven motor function along actin and microtubules are myosin and kinesin/dynein, respectively (see later).

Recent observations by Seiler et al. (1999) suggest that significant retrograde transport, at times exceeding the forward traffic of vesicles occurs in the vicinity of the hyphal tip. Thus, transport of compounds absorbed from the environment, or recycling of cellular proteins and other components from the hyphal tip region are also key processes involved in hyphal elongation. The efficacy of vesicle transport is much dependent on the proper function of nascent and mobile cytoskeletal elements. Actin microfilaments form longitudinally oriented cables and fine filaments in the cytoplasm extending, in some cases, to the apical dome and they are also associated with the Spitzenkörper. Microtubules are longitudinally oriented and they do not usually extend into the apical dome. Interestingly, not every case of functional disruption of cytoskeletal elements (by genetic alteration or drug treatment) results in measurable impairment of vesicle traffic or hyphal tip growth (Yamashita and May 1998). Furthermore, it appears that the organization of microtubules, as are the cytoskeletal motor complexes may well be diverse in different filamentous fungal species (and, apart from the structural conservation of the core elements, are also functionally diverse from yeasts).

Microtubules are involved in the formation of the mitotic spindle, intracellular transport of secretory vesicles (Howard 1981), positioning of the Spitzenkörper (Riquel et al. 1998), and organelle movement (Steinberg and Schliwa 1993). The initiation of microtubule polymerization (which involves the structurally-related α and β tubulin proteins) is determined by spindle pole bodies (microtubule organization centers) (Heath 1994). In vegetative hyphae, another tubulin, designated γ-tubulin (which is slightly less structurally similar to α and β tubulin than they are to each other) is present only in the spindle pole bodies. These spindle pole bodies are a critical determinant in the establishment of microtubule polarity and the concomitant direction of cargo transport within the hyphal cell.

Kinesins: The presence of members of the kinesin superfamily (comprised of the "founder" family of conventional kinesins along with kinesin-like proteins) in fungi is well documented. Lee and Plamann (2001) have described the structural and functional attributes of fungal kinesins (and other cytoskeletal components). The role of conventional and kinesin-like proteins in vesicle transport towards the hyphal tip as well as mitochondrial and nuclear movement have been demonstrated, yet their function in different filamentous fungi may vary (Lee and Plamann 2001; Steinberg and Schliwa 1995; 1996).

Dynein: Dynein and its accompanying multisubunit component, known as dynactin have been implicated to be involved in vesicle trafficking and mitosis. The multisubunit dynein/dynactin motor is complex and all indications are that their structure is highly similar to those present in higher eukaryotes. The complex is involved in retrograde transport, vesicle movement associated with the endocytic pathway, formation of the endoplasmic reticulum network, organization of the Golgi apparatus, and formation of the mitotic spindle (Karki and Holzbaur 1999). Plamann et al. (1994) have analyzed the abnormalities in nuclear distribution associated with defects in cytoplasmic dynein and have proposed models for the possible mechanisms by which the complex is involved in nuclear migration.

6 REGULATION OF ELONGATION/ BRANCHING

Hyphal elongation and branching are fundamental processes that define the morphology of filamentous fungi. The efficacy and success of these processes are essential for the outreach and acquisition of nutrients from the environment and in many cases, are part of the proliferative and reproductive cycle. The formation of the common cylindrical cell extension/branches by the polarized synthesis of new membrane and cell wall material must be both temporally and spatially regulated. Though information concerning the regulation of hyphal growth is accumulating, this aspect of hyphal cell biology is still in its infancy and a comprehensive picture of the network of processes involved has yet to be obtained.

The use of genetics has been instrumental in identifying and analyzing factors involved in the regulation of polar growth in both yeasts and filamentous fungi. The significant morphological differences between yeast and filamentous fungi suggest that in addition to the common basic machinery involved in establishment, maintenance, and changes in cell polarity, filamentous fungi may have additional or alternate regulatory pathways and are most likely to have more downstream elements (as required by their more complex

morphology) governed by the regulatory systems involved. A case illustrating a significant difference in a key regulatory pathway involved in polar growth of yeasts and filamentous fungi is described later.

Mutants that exhibit abnormal morphology of hyphal growth have served for identifying some of the genes involved in polar extension/branching of hyphae and the linkage between cell polarity and hyphal extension. Bruno et al. (1996) analyzed a temperature-sensitive *Neurospora* mutant (*mcb*) that exhibited apolar hyphal growth, which was not restricted to the apical cell of the hyphal filament. The mutant was shown to be defective in the gene encoding the regulatory subunit of cAMP-dependent protein kinase (PKA). The fact that the PKA pathway is involved in the regulation of polarized hyphal growth (the mutant is also defective in septum localization) in *N. crassa* is consistent with reports on the involvement of an activated PKA pathway in developmental switches that affect growth polarity in *Ustilago maydis* and *Magnaporthe grisea* (filamentous growth and proper appresorium formation, respectively). Later events in the penetration and infection processes of these fungi are stimulated by a mitogen-activated protein (MAP) kinase cascade, presumably through a MAP kinase module that may respond to the cAMP signal (see Chapter 4).

In contrast, even though an increase in PKA activity in *S. cerevisiae* has morphological consequences, these do not really involve cell polarity. This difference may reflect the different nature of growth of filamentous fungi, in which septation/cell separation is not linked with polar elongation (yet involves "cross-talk" between mechanisms regulating cell size and cell cycle). The comprehensive mechanistic involvement of PKA in regulation of polar growth and other cellular processes has yet to be obtained; most likely it involves the complex regulation of PKA expression itself, the interaction between PKA and its substrates, and interactions between PKA and other regulatory pathways.

A variety of hyperbranching mutants have been obtained in different filamentous fungi. The genetic dissection of these mutants is at its infancy. An example of an immediate need to understand such a mutant was the appearance of a hyperbranched colonial mutant of *Fusarium graminearum* strain A3/5, used to produce Quorn® myco-protein. The unexpected increase in the population of these colonial mutants in continuous flow cultures suggested a selective advantage over the sparsely branched wild type strain. The apparent advantage is an altered capability of glucose metabolism. Thus, the hyperbranching phenotype is due to a pleiotropic effect of the mutation in the colonial strain (Trinci et al. 1999). The influence of nutrient sources and the availability it has on colony morphology, as well as the effects paramorphogens have on fungal growth, and the means available to study the growth of the fungal colony have been recently reviewed by Olsson (2001).

The analysis of *Neurospora* colonial temperature sensitive (*cot*) mutants has yielded information concerning elongation and branching. Close examination of these mutants has clarified the fact that not all hyperbranching events are identical in rate of the elongation/branching frequencies or morphology of the branching pattern. Thus, it has become clear that diverse factors can result in considerably similar alterations in gross morphology while the mechanisms involved in imposing these changes can be very diverse, thus requiring much additional investigation.

At the permissive temperature, *cot-1* grows in a manner almost indistinguishable from that of the wild type, yet when shifted to the restrictive temperature hyphal elongation ceases concomitant with a massive induction of hyphal branching (Collinge et al. 1978; Yarden et al. 1992). The newly formed hyphal tips are unable to continue elongating at the restrictive-temperature, but returning the culture to the permissive-temperature results in the rapid restoration of normal hyphal growth. The *cot-1* gene has been cloned and, based on the deduced COT1 amino acid sequence it encodes a Ser/Thr-specific protein kinase that can be membrane associated (Gorovits et al. 1999; Yarden et al. 1992). Furthermore, evidence for a linkage between branching and fungal cytoskeleton assembly and function (Plamann et al. 1994) has set the stage for further analysis of the regulation and interaction of components governing the polarity of fungal filament growth.

Ser/Thr kinases that are highly similar to COT1 have been identified in other fungi (Buhr et al. 1996; Verde et al. 1998) and many higher eukaryotes, including *Candida elegans*, *Drosophila*, and mammals (Gorovits et al. 1999). The COT1 is highly similar to the product of the human *DM* kinase gene, which when mutated can cause myotonic dystrophy (Mahadevan et al. 1993). The common feature of most cells with alterations in their COT1-related kinase is abnormal morphology and observable defects in their polarity.

In *N. crassa*, COT1 kinase is essential, as insertional inactivation of the *cot-1* gene is lethal (Yarden et al. 1992). Lauter et al. (1998) have demonstrated that *cot-1* transcription is photoregulated. Using co-imunoprecipitation with antiCOT1 antibodies, Gorovits et al. (1999) have suggested the feasibility of a physical interaction between COT1 kinase and the catalytic subunit of type 2B phosphatase (calcineurin). Interestingly, a reduction in calcineurin activity (via antisense expression of the *cna-1* gene) also results in reduced hyphal elongation rate accompanied by hyperbranching (Prokisch et al. 1997). It is not surprising that different kinases/phosphatases are involved in hyphal elongation/branching, as they are involved in almost every cellular process. If and how the various components of different signal transduction pathways interact in filamentous fungi is far from being fully understood. The regulation of hyphal elongation/branching is probably complex, and involves a variety of signal transduction pathways. In addition to these pathways, it is conceivable that master regulators of gene expression involved in hyphal growth are involved. One such example may be the B genes in *U. maydis* (Brachmann et al. 2001). Another potential regulator of hyphal branching is the *pah1* homeobox gene of *Podospora anserina* (Arnaise et al. 2001).

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Overall, even with the abundance of colonial mutants isolated from a variety of filamentous fungi, the understanding of the genetic basis of these morphological abnormalities remains scarce (Turner and Harris 1999). Nonetheless, the availability of so many mutants may well prove an asset for expansion of the current attempts to functionally analyze genes involved in hyphal elongation and branching. Elucidation of this process is important not only for satisfying the basic need of understanding such a fundamental process, but also as a starting point for rationally intervening in this process. Curbing the proliferation of pathogens, or enhancing growth and morphogenesis in industrial strains, would both benefit once a clearer mechanistic picture of these events is obtained. Much hope has been put on the expected increase in secretion from hyperbranching mutants. The components involved in secretion in filamentous fungi are highly similar to those found in yeasts and higher eukaryotes. However, in contrast to other organisms, the hyphal cell with its polar apical extension creates a unique morphological arena for the process of secretion. Much focus has been put on understanding secretion at the apical zone of growing hyphae (Conesa et al. 2001). However, even though a link was proposed between growth and secretion at hyphal tips in filamentous fungi (Wessels 1993), the increase in hyphal tips per unit of biomass does not necessarily result in a parallel increase in secretion of extracellular enzymes (Conesa et al. 2001; Trinci et al. 1999).

7 STUDYING THE BIOLOGY OF THE HYPHAL CELL

7.1 Biochemical Genetics

A filamentous fungus was used to open the door to biochemical genetics (Beadle and Tatum 1941). Since that bridge between genes and their biochemical function was formed, the biochemical genetics approach has become part and parcel of almost every analytical venture in biology. Thus, understanding biochemical pathways and the genetic basis of the structural and regulatory elements from which they are comprised is still a common and efficient strategy to advance our understanding of any cellular process. The haploid nature of many fungi (during most of their life cycle), the availability of mutants (or the means to produce them) and the development of methodology by which almost any fungal species can be made amenable to DNA-mediated manipulations provide a likely assurance that biochemical genetics approach, along with others, will continue to be in the mainstream of fungal biology research. The availability of complete fungal genomes has become a reality (see later) and the high-throughput technology encompassing the genomics and postgenomics era are certain to contribute immensely to the future advances in understanding fungal biology. Nonetheless, the power of classical genetics linked to biochemistry has not diminished and will most likely continue to serve as a solid foundation for resolving many fundamental questions in fungal biology.

7.2 Cell Imaging

Macroscopic and microscopic observation has always been a key element in the analysis of the fungal cell. Thus, as early as the third quarter of the 17th century Sir Robert Hooke, utilizing his optical magnification apparatus described the fungal cell (amongst those of other organisms he observed). Microscopy has accompanied the mycologist ever since and still provides an important means in studying fungal biology. The availability of reporter systems along with molecular transformation techniques and recent advances (as well as traditional technologies) in microscopy (light, fluorescent, confocal, and electron) allows the detailed probing of events at the cellular level. Measuring the relative intracellular ion concentrations in specific areas of hyphae (and other fungal structures) is possible by X-ray microanalysis procedures (Connolly et al. 1999). Immunocytochemical techniques (utilizing fluorescent or gold particle-labeled antibodies) are accessible and have been integrated into a growing number of study systems for localization studies, colorimetric visualization, and measurement of reporter gene expression in fixed or live tissues (e.g., β-glucoronidase or green fluorescence protein systems). Perhaps the most exciting current developments are the improvements in live fungal cell imaging. The use of classic dyes that are sensitive to changes within the living cell (e.g., pH, specific ions, reactive oxygen species, etc.) along with dyes that are membrane selective (e.g., FM4-64) and in combination with fluorescent and confocal microscopy will certainly open new avenues of research that will shed light on the cellular biology of the hyphal cell (Fischer-Parton et al. 2000; Heath 2000).

7.3 High-Throughput Analyses (The "X-omics" Age)

The first fungal genome to be sequenced in its entirety was that of *S. cerevisiae* in 1997. Since then, several fungal genomes have been sequenced (some more than once) by private ventures. Recently, the first complete sequence of a filamentous fungus, *N. crassa*, has been made available to the public (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). The *Aspergillus nidulans* genome is also available, with restrictions (http://www.fgsc.net/aspergenome.htm). Others are likely to follow soon, as public efforts for sequencing the genomes of several additional filamentous fungi are under way. These include *Cryptococcus neoformans* (http://www-sequence.stanford.edu/group/C.neoformans/), *M. grisea* (http://www.riceblast.org/), *A. fumigatus* (http://www.sanger.ac.uk/ Projects/A_fumigatus/), *Candida albicans* (http://sequence-www.stanford.edu/group/candida/index.html), *Phanerochaete chrysoporium*

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(http://www.jgi.doe.gov/ programs/whiterot.htm), and others. The sequencing of entire genomes will provide the required information for enhancing the ability to perform high-throughput analyses of gene structure and function (Sweigard and Ebbole 2001). The age of structural and functional genomics, transcriptomics, proteomics, metabolomics, and additional categories and subcategories has begun. The prospects for utilizing the new data and technological tools for understanding the molecular basis of cellular events in the hyphal cell are enormous (see Chapters 12 and 17). It will now be easier to combine reductionist approaches (e.g., analysis of single gene/protein function) with some of the more holistic approaches involving the concurrent study of several components affecting a specific trait.

8 CONCLUSIONS

Most fungi posses most of the common cellular features shared by other eukaryotes. However, the distinct shape and some of the metabolic features of the hyphal cell require that the activities of the fundamental, conserved, cellular machinery be amended with the unique structural and functional elements required to maintain the hyphal cell and support its growth. Thus, identifying the structural components (common and unique), determining their function and elucidating the regulatory mechanisms responsible for the concerted interactions between subcellular components on the one hand and the intact organism and its environment, on the other, are instrumental for our efforts to intervene with the activities of these fascinating and diverse organisms.

The advances made in dissection of individual components of the hyphal cell (e.g., cell wall, organelles, and septa) are significant. The combination of biochemistry, genetics (classical and molecular), and microscopy have yielded impressive results in the process of identifying many of the components involved, determining their significance in hyphal growth, and associating them with the topography of the hyphal cell. However, we are still challenged with a need to understand many of the mechanistic aspects of hyphal cell biology. At times, the difficulties involved in the analysis of these aspects are shared by those studying other eukaryotic cells (e.g., function of membrane-associated proteins, organization and function of cytoskeletal elements, and regulation of the cell cycle, etc.) and conceptual or technological breakthroughs in the field of cell biology are likely to be of significant value to fungal biologists. However, understanding the unique aspects linked to fungal morphology and growth, and intervening in these processes, depend on advances in mycological research in general and the in-depth analysis of fungal species of interest.

The outcome of the current effort invested by members of the fungal biology community in advancing the fields of fungal genomics and proteomics may well prove to be an invaluable stepping-stone in better understanding the hyphal cell. Comparative genomics (among filamentous fungi and between fungi and other organisms) will assist in identifying "missing links" in conserved pathways, while at the same time point to unique proteins in fungal species. Nonetheless, at the end of the day, classical conceptual approaches used in cell biology research during the past decades will be required in order to answer the fundamental questions relating to growth and development of the hyphal cell.

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2

Protoplast Isolation, Regeneration, and Fusion in Filamentous Fungi

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1 INTRODUCTION

In fungi, protoplasts can be defined as spherical cells whose chitinous cell walls have been removed by appropriate digestive enzymes (Bachmann and Bonner 1959). Based on the considerable literature that has accumulated over the past 40 years, protoplast studies in fungi can be divided in two main sections: (a) production of stable, complete protoplasts, and (b) use of protoplasts, for example, to obtain intact chromosomes, as partners in fusion studies, and as recipients of exogenously introduced DNA (a process termed as transformation). The successful applications of such protoplast manipulations has led to a better understanding of many biochemical, physiological, and genetic phenomena in fungi.

The technique of protoplast fusion in particular, continues to contribute positively to our understanding of fungal biology in topics ranging from secondary metabolism to vegetative incompatibility (discussed in Section 8). However, to the best of our knowledge, the topic of protoplast fusion has not been reviewed in recent times. Another technique involving protoplasts, that of DNA transformation, has also been very useful in fungal research, particularly in those fungi where sexual crosses are not feasible (imperfect fungi). For example, DNA-mediated transformations were instrumental in deciphering the molecular regulation of aflatoxin biosynthesis in *Aspergillus flavus* and *A. parasiticus* (Horng et al. 1990; Payne and Woloshuk 1989; Trail et al. 1995; Woloshuk et al. 1989; Yu et al. 1995). Since the topic of fungal transformation has been reviewed in many current and comprehensive reviews (Hamer et al. 2001; Punt and van den Hondel 1992; Ruiz-Diez 2002; Mullins and Kang 2001; Shishido 1994), this chapter will not cover this topic, but will focus on protoplast fusion. The contents of this chapter include first a review of the salient features of protoplast isolation, followed by a discussion of fusion studies specifically in filamentous fungi in the context of fungal genetics. Where applicable, both the advantages and the limitations of the protoplast fusion technique will be highlighted. This article is targeted for those relatively "new" to the world of protoplasts, but should also serve as a source of literature to the more established fungal researchers.

2 HISTORICAL PERSPECTIVE

The earliest studies on the formation and development of fungal protoplasts were reported in *Neurospora crassa* (Bachman and Bonner 1959; Emerson and Emerson 1958) and *Fusarium culmorum* (Rodriguez-Aguirre et al. 1964). However, successful application of these protoplasts in biochemical or genetic studies would depend on the optimization of several parameters influencing their yield and stability including the effectiveness of the digestive enzymes and the ability of the protoplasts to regenerate (discussed later).

Initially, protoplasts were used mainly to isolate intact organelles and to prepare cell lysates for biochemical analyses [reviewed by Villanueva and Garcia (1971)]. Soon after, the first reports on spontaneous protoplast fusion appeared for *Geotrichum candidum* (Ferenczy et al. 1974) and *A. nidulans* (Ferenczy et al. 1975). Almost simultaneously, Anne and Peberdy (1975) reported their results on the induced protoplast fusion in *Penicillium chrysogenum* using polyethylene glycol (PEG) MW 6000 as a stimulant. Around the

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same time, the first report of DNA mediated transformation for a filamentous fungus appeared in *N. crassa* (Case et al. 1979). Since then, protoplasts have continued to serve as valuable tools in a wide range of basic and applied research projects.

3 PROTOPLAST ISOLATION

The basic approach for preparation of protoplasts from filamentous fungi has changed little over the years. Essentially, it involves the treatment of the hyphae with enzymes that will digest the cell wall contents. Since, the spherical protoplasts are osmotically fragile, organic or inorganic stabilizers must be added to the medium to help maintain their morphology. The major factors influencing the process of protoplast isolation are summarized below.

3.1 The Fungal Cell Wall and Digestive Enzymes

The fungal cell wall is a hardy structure that gives the cell its shape, protects it under the normally hypotonic environments, plays an important role in cell–cell interactions due to the presence of surface antigens, and acts as a site of extracellular enzymes with hydrolytic or metabolic activities (Farkas 1985).

In general, a fungal wall is composed of 70% or more polysaccharides (homo and hetero) and variable amounts of proteins, lipids, and melanins (Bainbridge et al. 1979; Bartnicki-garcia 1968; Rosenberger 1976). The polysaccharides are further classified into skeletal polysaccharides composed mainly of chitin and R-glucan, and wall-matrix polysaccharides composed mainly of protein–polysaccharides (Frakas 1985). Ultrastructure studies on *Pythium acanthicum* and *N. crassa* cell walls have shown that the skeletal polysaccharides are embedded in an amorphous matrix of protein–polysaccharide complexes such that the outer surface of the wall is usually smooth while the microfibrils of the skeletal polysaccharides are visible on the inner layer (Burnett 1979; Sietsma et al. 1975). The general cell wall composition, along with the functions of its major components are shown in Figure 1.

Since, protoplasts are able to form cell walls *de novo*, an understanding of the sequence of events that leads to this phenomenon is essential. Although advances in this area have been made (see Section 4), details of the assembly of the wall components and the genetic factors that are involved in the regulation of wall synthesis remain poorly understood.

With respect to cell wall lysis, for several years, the snail *Helix pomatia* digestive juice preparation was used to obtain protoplasts in *N. crassa* (Emerson and Emerson 1958), *A. nidulans* (Ferenczy et al. 1975) and *G. candidum* (Ferenczy et al. 1974). Mycolytic enzymes obtained from bacterial [*Arthrobacter*, *Bacillus*, and *Streptomyces* (Doi et al. 1971; Kitamura et al. 1974)] and fungal [*Trichoderma* (Benitez et al. 1975a, b; de Vries and Wessels 1973) and *Aspergillus* (Isaac and Gokhle 1982)] sources have also been used to produce

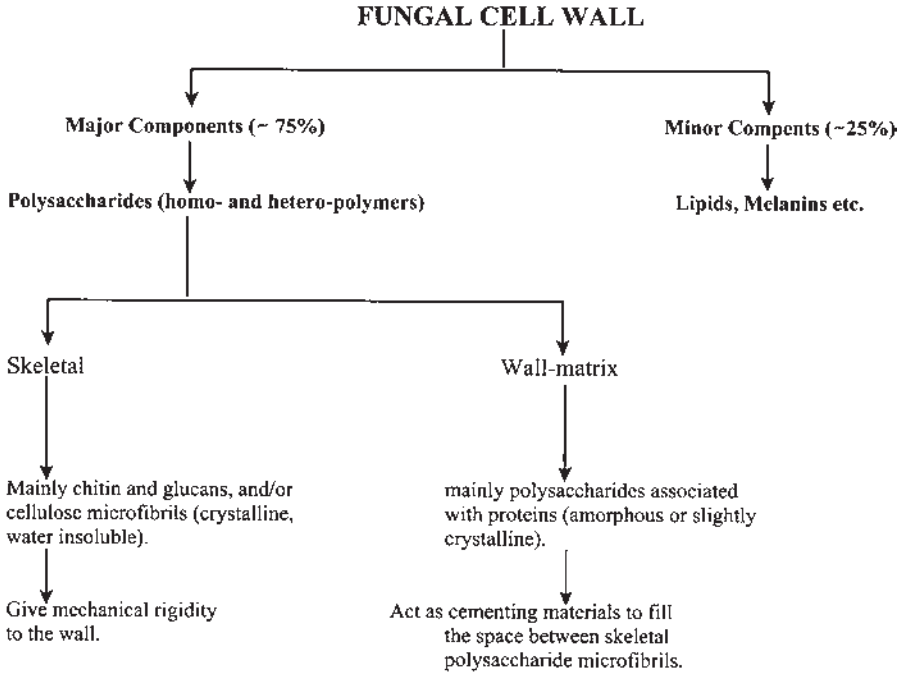

Figure 1 Schematic representation of the fungal cell wall [summarized from Farkas (1985)].

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fungal protoplasts. Regardless of the sources, these enzyme preparations have polysaccharases as a common activity, with differing amounts of individual subtypes like glucanase I and II, cellulase, mannanase, and chitinase [reviewed by Peberdy (1985)]. Often, the enzymes are not pure and are present as a mixture of polysaccharase, protease, and lipase activities (Scott and Schekman 1980; Villanueva and Garcia 1971). Although many laboratories preferred making their own lytic preparations, these preparations were laborious, varied in their lytic activities and gave variable yields of protoplasts (Villanueva and Garcia 1971). In 1981, findings of a comprehensive study that involved the comparison of activities of several commercial polysaccharases (Novozym 234, Cereflo 200L, cellulase, helicase, chitinase, D-glucuronidase, and D-glucanase) for their ability to liberate fungal protoplasts was reported (Hamlyn et al. 1981). It was found that while enzymes individually, or in combination, varied in their ability to produce protoplasts depending on the type of fungus, in general, Novozym 234 (Novo), an enzyme mixture of chitinase, cellulase, and protease activities from *Trichoderma* species, was the most effective and produced sufficient numbers of protoplasts in 2–3 h (Hamlyn et al. 1981). Novozym 234 is also available from other commercial sources like Sigma and continues to be widely used for fungal protoplasting [for example in *P. chrysogenum* (Hong and Robbers 1985), *A. flavus* (Keller et al. 1992), *A. nidulans* (Bird and Bradshaw 1997), and *Lentinus lepideus* (Kim et al. 2000a)].

3.2 Age of the Mycelium and Pretreatment of the Organism

Although fungal protoplasts can be obtained from ungerminated fungal spores (Bos and Slakhorst 1981; Cheng and Belanger 2000; Moore and Peberdy 1976), most laboratories use mycelia as the starting material mainly because the spore wall is highly resistant to lysis (Peberdy 1979a). With respect to hyphal age, results vary from fungus to fungus but in general, 18–48 h old mycelia, corresponding to the early to mid-exponential growth phase result in the best protoplast yield (Peberdy 1979b). Occasionally, stationary-phase mycelia also yield protoplasts (as a second peak after the log-phase protoplasting) as was the case in *P. chrysogenum* (Anne et al. 1974), which suggested the possible involvement of an endogenous lytic activity. More recently, it has been reported that older hyphae not only resulted in release of fewer protoplasts, but the resulting protoplasts tended to be fragile and contained large vacuoles (Harman and Stasz 1991). Based on the early observations on protoplast formation in *Fusarium* species (Lopez-Belmonte et al. 1966), the hyphal walls swell at specific points before release of the protoplasts. Presumably these points are the initial sites of the enzyme attack and are most accessible when the hyphae are actively growing. In any case, the log-phase cultures also require lesser amounts of the lytic enzyme and shorter incubation times to obtain protoplasts so most studies report the use of 18–48 h old mycelia.

Certain media additives have been helpful in that, when the fungi are preincubated in these media, higher numbers of released protoplasts are obtained. These include reducing agents (e.g., dithiothreitol, 2-mercaptoethanol, and sodium thioglycolate), detergents (e.g., triton X-100 and deoxycholate), and chelating compounds (e.g., EDTA), [reviewed by Davis (1985)]. The thiol compounds may cleave the disulfide linkages in the cell wall (Peterson et al. 1976), thus facilitating the activities of the digestive enzymes, while the detergents may be involved in the removal of the lipid components of the cell wall (Davis 1985).

3.3 Osmotic Stabilizers, Temperature, and PH

Osmotic stabilizers are needed to maintain the protoplast morphology, which is otherwise maintained by only a cell membrane. In the past two and a half decades, a wide range of inorganic salts (KCl, $CaCl_2$, $(NH_4)_2SO_4$, NH_4Cl, $MgSO_4$, NaCl) and organic compounds (sugars and sugar alcohols) have been used in this capacity [reviewed by Davis (1985) and Villanueva and Garcia (1971)]. The experimental data on a range of systems has suggested that in general, inorganic salts (in 0.6 M range) work better for filamentous fungi while sugar and sugar alcohols are more suitable for yeasts (Peberdy 1979b). Further, depending on the enzyme preparation, some stabilizers may actually assist in the wall lysis. For example, a combination of $CaCl_2$ and KCl greatly enhanced the protoplast release from *A. niger* and *A. fumigatus* when chitinase was included as the lytic enzyme, but if $CaCl_2$ alone was used as a stabilizer, the protoplast yield actually decreased (Thomas 1981; Thomas and Davis 1980).

Other variables like pH and temperature also influence protoplast production based in part, on their influences on the activities of the lytic enzymes. As with other influencing factors, optimum pH and temperatures have to be determined experimentally for each fungus, but it appears that a range of 20–40°C work well with most filamentous fungi (Davis et al. 1977; Petrini 2001; Thomas 1981). Temperatures higher than 40°C may inhibit the activity of the lytic enzymes while lower temperatures may affect the protoplast membrane stability (Kovac and Subik 1970). With regards to the pH of the incubation medium, in general, acidic environments (pH below 4) tend to decrease the lytic activities of the digestive enzymes although optimum pH varies from one organism to another. Additional details on these factors and their possible side-effects on protoplast metabolism have been discussed by Davis (1985) and Peberdy (1979b).

4 PROTOPLAST REGENERATION

This process refers to the ability of the protoplasts to revert back to the normal filamentous form by first synthesizing the

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cell wall. While producing protoplasts is a crucial first step, the success of any experiment involving protoplasts largely depends on whether they can regenerate through cytokinesis and form visible colonial growth on osmotically balanced solid media. Over the years, most studies on the dynamics of cell wall regeneration have been conducted in yeast, in part because of the ability of some to undergo the yeast/mycelial (Y/M) shift, making comparative studies possible. Such studies (discussed later) have aimed at understanding the specific events of wall polymer synthesis in regenerating protoplasts and the regulatory factors that control their sequence. Data from some early studies indicated that the first structure to be assembled on the surface of the regenerating protoplasts is the microfibrillar network of chitin and glucan synthesized probably by enzymes associated with the plasma membrane (Van der Valk and Wessels 1976; 1977), and this event is usually followed by the assembly of the amorphous matrix (Necas 1971; Peberdy 1979a). In another study on *Candida albicans*, the zymogenic stage (when protoplasts are getting formed) was associated with incorporation of higher amounts of chitin synthetase in the cell membrane (Elorza et al. 1983). The protein synthesis was initiated within the first 40 min and after a few hours, chitin was the most abundant polymer in the aberrant wall of the regenerating protoplasts (Elorza et al. 1983). More recently, quantitative measurements of the fluorescence emitted by individual *C. albicans* protoplasts indicated that chitin and mannoproteins are among the first components to be laid down on the regenerating protoplasts followed by the glucans (Rico et al. 1997). In a related study (Nishiyama et al. 1995), similar results were obtained based on electron microscopic observations using a fluorescent dye (Fungiflora Y) that specifically binds to beta-linked polysaccharides. In this study, fluorescence was detected on multiple sites of the fresh protoplasts within 15 min of incubation; by 1 h the entire structure was fluorescing, and after 24 h of incubation, the reverted cells were capable of proliferating (Nishiyama et al. 1995). On a molecular level, in the fission yeast, experiments involving two novel protein kinase C homologs *pck1+* and *pck2+* have shown that the *pck2+* gene is essential for protoplast regeneration, but *pck1* is not (Kobori et al. 1994). In another recent study, mRNA expression levels of 38 stress response and cell wall protein genes from *Saccharomyces cerevisiae* were investigated during spheroplast regeneration by Braley and Chaffin (1999); a spheroplast being defined as a cell which may have portions of the cell wall still attached (Davis 1985). It was observed that although the expression of some of these genes did increase between 5- and 10-fold during attempted regeneration, their transcription was not sufficient to initiate the replacement of the cell wall (Braley and Chaffin 1999). From these recent examples, it is evident that the genetic basis of protoplast regeneration is a series of complex interactions of several factors and a better understanding of this process will have to await further research.

5 PROTOPLAST FUSION

The process by which the protoplast membranes fuse (plasmogamy) to have a common, multinucleate cytoplasm is called protoplast fusion. As mentioned earlier, protoplast fusion can occur naturally but the frequency is low and so is the yield of the fusants. With respect to induced fusion, perhaps the most important development was the demonstration by Anne and Peberdy (1976) that PEG, a known fusagen of plant protoplasts (Kao and Michaylink 1974) can successfully be adapted for that purpose in fungi as well. The PEG is thought to be involved in forming the hydrogen bonds (by itself or in the presence of Ca^{+2} ions) between the cell membranes (Peberdy 1979b). To determine the optimal conditions for induced protoplast fusion, a comprehensive study was undertaken by Anne and Peberdy (1975). Using complementing auxotrophs of *P. chrysogenum*, they reported that best results were obtained when the PEG (MW 6000) solution was at least 20% (w/v) and was supplemented with Ca^{+2} (in form of 10 mM $CaCl_2$) under alkaline conditions. The significance of this report is reflected in the fact that even today, almost every laboratory utilizes these conditions with occasional modifications for successful protoplast fusions (Brauer and Robbers 1987; Couteaudier et al. 1996; Martinkova et al. 1990; Ogawa et al. 2000).

It should be noted that protoplast fusion can also be accomplished using electric currents in the form of DC pulses (Fukuda et al. 1995; Zimmerman 1982) but this technique has not been very widely used.

In experiments involving PEG, the fusion events are random and if two parents are used, fusants can be formed which are like (partner protoplasts are from the same parent) or mixed [partner protoplasts are from two different parents (Peberdy 1987)]. Further, since the numbers and the types of nuclei vary in the protoplast aggregates, the proportion of the two parental types of nuclei is thought to most influence the stability and development of the first generation of fusion products, the heterokaryons (Peberdy 1987). Once the fusion products are obtained, they are recovered and analyzed by using several selective techniques as discussed in the later sections.

6 THE PARASEXUAL CYCLE AND PROTOPLAST FUSION CONNECTION

Parasexual cycle is one of the ways by which fungi can exchange genetic information. In fact, in the imperfect fungi which lack a sexual phase, it is the only known means for genetic recombination.

The parasexual cycle was first discovered in *A. nidulans* (Roper 1952) and shortly after, was shown to occur in *P. chrysogenum* (Pontecorvo and Sermonti 1953) and the pathogenic fungus *Fusarium oxysporium* (Buxton 1956). It consists of three basic stages: heterokaryosis, diploidization, and recombination through mitotic crossing over or

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haploidization. Heterokaryosis is the anastomosis or fusion of the hyphae. The schematic representation of formation of protoplast fusants and their analysis is shown in Figure 2. In a laboratory setting, this process can be observed using two compatible haploid strains differing in one or more characteristics that serve as genetic markers. So, for example, if the marker is that of a nutritional requirement (different one in each parent), neither parent can grow on a minimal medium (MM). However, if a heterokaryon forms due to hyphal fusion, it can grow on the MM. This is because it contains the nuclei of both parents which complement each other's defect. In this instance, the MM serves as the selective medium. Often, the auxotrophic crossing parents also carry different spore color markers. Although not essential, spore colors are useful markers, because if the two parents produce spores of contrasting colors, the heterokaryon is seen as a mixture of both spore colors (Bennett 1979). Similarly, among the auxotrophs some are easier to isolate and hence more often used. For example, mutants defective in the genes of nitrate assimilation pathway like the nitrate reductase (*nia*D) mutants can be easily isolated, based simply on their resistance to chlorate without the need of any mutagen (Cove 1976). Auxotrophic nutritional markers, however, can be "leaky" (a small amount of the nutrient is still made by the

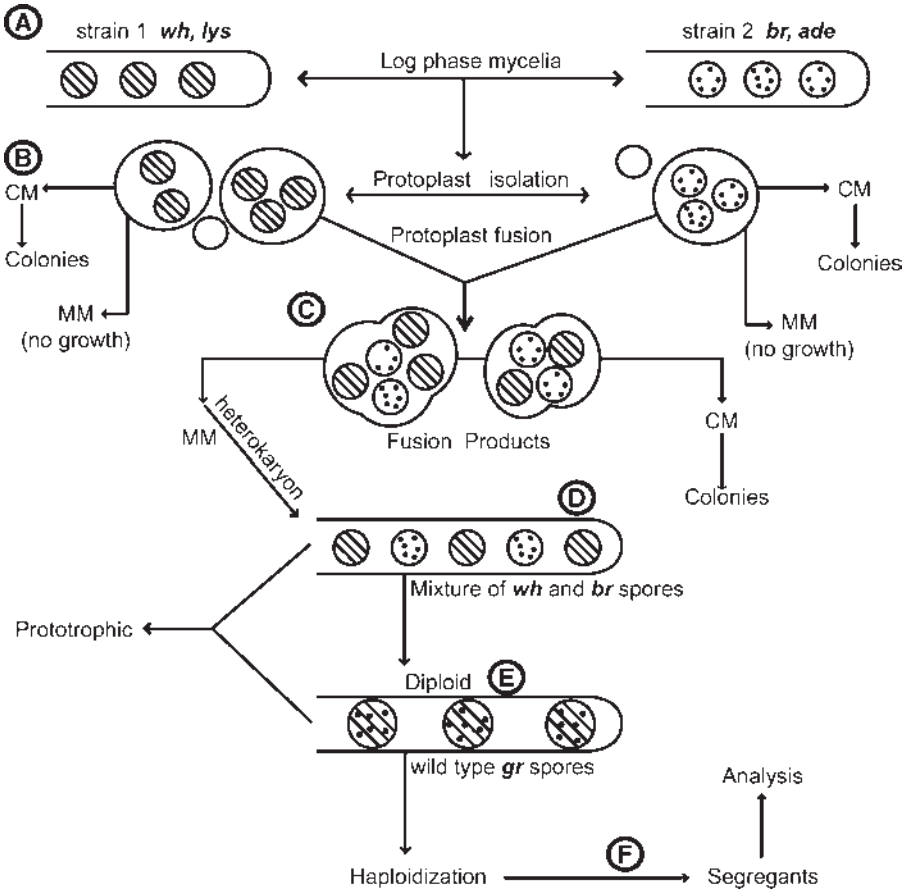

Figure 2 Schematic representation of fungal protoplast fusion and general scheme of fusion progeny analysis. A: Strain 1 is auxotrophic for lysine (*lys*) and has white (*wh*) spores. Strain 2 is auxotrophic for adenine (*ade*) and has brown (*br*) spores. Both strains are derivatives of wild type (WT) prototrophic cultures that have green (*gr*) spores. B: Heterogeneous protoplasts of varying sizes are obtained from log-phase mycelia of strains 1 and 2. Neither type of unfused protoplasts grow on agarbased MM because of their respective auxotrophic requirements, but growth is obtained on agarbased nutritionally CM. C: Protoplast fusion products are formed as aggregates containing varying numbers of parental nuclei. Growth occurs when these fusants are plated on CM and MM. Fusion frequency is defined as a ratio of number of colonies on MM/number of colonies on CM. D: A heterokaryon carrying the two types of parental nuclei. On MM, the heterokaryon will grow as a prototrophic mixture of *wh* and *br* spores, with the two types of parental nuclei complementing each others' nutritional deficiencies. E: Nuclear fusion in the heterokaryon gives rise to prototrophic diploids that have the wild type (*gr*) spore color. F: Haploidization of the diploids gives rise to segregants in form of sectors, which can be purified and further analyzed. [Based on Anne and Peberdy (1976), Bennett (1979), Peberdy (1979b), and Scott et al. (1979). Additional details are given in the text].

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fungus under certain growth conditions; Bennett 1979; Hsieh et al. 1976) and sometimes, can negatively influence the growth or secondary metabolite production of the fungus (Ferenczy et al. 1987; Kikuchi 1980). In response, other genetic markers have been used, including those for resistance to antibiotics and fungicides (Tudzynski et al. 1982) which aid in the identification of the heterokaryon.

In the second step of the parasexual cycle, rare nuclear fusion in the heterokaryon produces the heterozygous diploid which is identified as a nonsectoring stable prototroph on MM with the wild type mycelial and spore color. During vegetative multiplication of the nuclei in the diploid, mitotic crossing-over events can lead to diploid segregants, or nondisjunction phenomena can give rise to haploid recombinant segregants. In this way, even in the absence of a sexual cycle, genetic exchange can occur (Papa 1978; Roper 1966; Scott et al. 1979).

The events that occur in most protoplast fusions parallel those in the parasexual cycle with one advantage. Fusion of the protoplasts can bypass the incompatibility barriers that exist at the hyphal level which, in the parasexual cycle prevent the formation of a heterokaryon (Dales and Croft 1977, and discussed later). Like in parasexual analysis, as long as there are appropriate genetic markers and tests in place to distinguish the heterokaryons vs. the diploids vs. the haploid segregants, protoplast fusion can serve as a powerful tool for a wide range of genetic studies. For instance, once the products of the parasexual cycle (diploids, haploids, etc.) are obtained, they can be analyzed for linkage studies, secondary metabolite production, virulence, and other characteristics under study (Bennett 1985; and as discussed in Section 8).

7 MOLECULAR MARKERS

For the past several years, molecular markers such as isozyme analysis, restriction fragment length polymorphism (RFLP), and rapid amplified polymorphic DNA have been employed to more accurately identify the recombinant progenies obtained from the postfusion events. Isoenzyme analysis is based on the ability of an enzyme to exist in various forms, which (depending on the difference in their electrophoretic mobilities) can be visualized as distinguishable bands when separated according to their charge distribution at a specific pH (Anne 1977; Stasz et al. 1988). Thus, once isoenzyme profiles for a particular strain are established, they can serve as efficient molecular markers to demonstrate the formation of a heterokaryon and/or to identify recombinant haploids (Anne 1977; Stasz et al. 1988).

Restriction fragment length polymorphism is based on the fact that relatively common differences in the DNA sequences exist in all organisms including filamentous fungi. Such DNA polymorphisms can be visualized by cutting the genomic DNA with restriction enzymes, separating the resulting DNA fragments by agarose gel electrophoresis and then performing Southern blot using the cloned gene or gene-fragment of interest as a probe (Hartl and Jones 2002). Using this technique, any process (e.g., mutation, recombination, retrotransposition, etc.) which yields different-sized DNA fragments can be visualized as a novel RFLP pattern. Among its many applications, this technique can be used to analyze DNA similarities of the samples. For example, recombinant DNA clones carrying sequences from *A. nidulans* and *N. crassa* were used to identify diagnostic RFLPs for members of the *A. flavus* groups (*A. flavus*, *A. parasiticus*, and *A. nomius*), and the fungi were resolved in three different categories based on the RFLP profiles (Moody and Tyler 1990). More recently, RFLP markers were isolated and tested for *A. fumigatus*, and the RFLP analysis revealed clear polymorphism in these otherwise closely related fungi, thus establishing their status as two distinct species (Parenicova et al. 2001). In the context of protoplast fusions, RFLPs can determine whether the genetic makeup of the fusion progeny is parental (RFLP pattern identical to one of the parents) or recombinant (RFLP pattern different from either parents). Specific applications are discussed in the following section.

Random amplified polymorphic DNA (RAPD) is a relatively new assay (Williams et al. 1990) which also detects DNA polymorphisms. However, instead of cloning a gene and conducting laborious Southern analysis for RFLP, it is based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequence. The polymorphisms of the primer DNA fragments (RAPD profiles) are then visualized simply by gel electrophoresis and ethidium bromide staining of the DNA (Durand et al. 1993; Williams et al. 1990).

8 PROTOPLAST FUSION APPLICATIONS IN BASIC AND APPLIED GENETICS

Often, a genetically well-understood organism is also the more commonly used in advanced studies. In the filamentous fungi, this has been true for *N. crassa* and *A. nidulans*; and the latter in particular, has been extensively employed for protoplast studies, some of which are discussed below.

8.1 Vegetative Incompatibility and Related Phenomena

Also referred to as somatic or heterokaryon incompatibility, this phenomenon can be defined as the inability of fungal mycelia to fuse, to form heterokaryons, and to exchange genetic information. Its occurrence is well documented in several *Aspergillus* species and is thought to be a mechanism for isolation within a species (Croft 1987). As a case study, in *A. nidulans*, wild isolates were classified into heterokaryon compatibility (h-c) groups and the compatibility differences shown to be under nuclear control (now called the *het* genes) as early as 1963 (Grindle 1963). Based on this classification, members within any one h-c group can readily form heterokaryons with each other, while members of different

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h-c groups do not form heterokaryons. Since, the *het* genes do not interfere with sexual reproduction, early studies based on their segregation patterns in the progeny of sexual crosses between members of differing h-c groups resulted in the demonstration of their heterogenic nature (Esser and Blaich 1973). However, such work was rather laborious, and the problem was overcome by the advent of protoplast fusion. By fusing protoplasts of vegetatively incompatible strains, heterokaryons were obtained which were then subjected to standard parasexual analysis in order to understand vegetative incompatibility (Dales and Croft 1977). Using the "master Glasgow strains" [which have eight scorable markers, one on each linkage group (McCully and Forbes 1965)] and members in other h-c groups like h-c B, h-c A, h-c R, and h-c Q as crossing parents, the following general scheme was employed by Dales and Croft (1983; 1990); Dales et al. (1983):

(a) Protoplasts of the two parental strains (having differing spore color and auxotrophic markers) were prepared, induced to fuse, and plated on osmotically stabilized MM. Appropriate controls were also set up to ensure that the results seen were indeed due to the fusion of the protoplasts and not due to back mutation or other phenomena. (b) Detailed observations were made regarding the phenotype of the presumptive heterokaryons especially with respect to growth patterns, amount of sporulation, and the color of the spores. Heterokaryon formation was confirmed by subculturing colonies on complete media (CM) and recovering parental phenotypes. (c) Presumptive diploids were isolated based on their wild type spore color (or sometimes a shade lighter than wild type spore color), the spore diameter (whether or not it was significantly larger than that of haploid conidia), and mycelial color. (d) Representative diploids were analyzed by haploidization analysis using the inducing agent benlate (Hastie 1970). (e) Analysis was done to determine the linkage group on which the *het* difference(s) resided. For example, pairs of haploid progeny identical for all nuclear markers except on the linkage group under question (say group 1) were subjected to heterokaryon compatibility tests. If the results were positive for heterokaryon formation for all (or almost all pairs), it was deduced that the original crossing parents did not have any *het* difference(s) on linkage group 1.

Using this approach, extensive data was obtained from all five compatibility test groups (h-c GL, h-c A, h-c B, h-c R, and h-c Q). A compilation and comparison of these results indicated that a minimum of eight and a maximum of 18 loci spread over five linkage groups were necessary to explain the compatibility relationships among the five h-c groups (Anwar et al. 1993).

Based on this work and related studies in other fungi like *Gibberella* (Adams et al. 1987) and *Cochliobolus* (Leach and Yoder 1983), it is quite evident that the vegetative incompatibility phenomenon is heterogenic, and having dissimilar alleles at any one of the several loci can make the strains incompatible. Further, based on the degree of such difference(s), "levels" of incompatibility may exist. In general, greater the relatedness between the two fungal partners, greater is the possibility that they are vegetatively compatible. Another generality—although protoplast fusion can force two incompatible strains to form a heterokaryon, frequency of diploid formation is not always high, and recovery of the haploid progeny may be slow. Finally, protoplast fusion cannot solve the postfusion vegetative incompatibility that may be due to other influences such as those of mitochondria and other cytoplasmic elements (Harman and Stasz 1991; Peberdy 1987).

Certain aspects of our work on strain degeneration in filamentous fungi with respect to secondary metabolite production (Kale and Bennett 1992; Kale et al. 1994) using aflatoxin producing *A. parasiticus* (an imperfect fungus) as a model system appear to be related to the phenomenon of heterokaryon incompatibility as follows: A collection of degenerate strains (designated *sec*−) which no longer produce any aflatoxins or its intermediates have been generated from the toxigenic parental strains simply by transfer of mycelial macerates (Kale et al. 1994). The *sec*− strains were found to be pleiotropic in nature in that, apart from loss of secondary metabolite production, they also displayed altered morphology and reduced sporulation compared to their *sec*+ parents (Kale et al. 1994). Since then, we have subjected the isogenic *sec*+/*sec*− pairs to exhaustive stability testing, biochemical, and molecular studies and have demonstrated that the *sec*− forms are highly stable (none have reverted back to the *sec*+ form), that they have intact aflatoxin pathway structural genes, and that there is no (or very little) expression of any of these genes including the *afl*R regulatory gene (Chang et al. 1993; Payne et al. 1993) in these *sec*− variants (Kale et al. 1996). Along with the ongoing molecular work, we have also conducted comprehensive parasexual analysis of the *sec*− strains (Kale et al. unpublished study). Results of parasexual crosses show that when *A. parasiticus sec*+ and *sec*− strains of contrasting spore color and auxotrophic markers are used in crossing, *sec*+/*sec*+ crosses yield heterokaryons and diploids with ease, *sec*+/*sec*− crosses generate heterokaryons and diploids with an intermediate level of success, and *sec*−/*sec*− crosses yield no viable heterokaryons and consequently no diploids even after 10 attempts and compensating for reduced spore counts of the *sec*− forms. The cytoplasmic inheritance test (Arlett et al. 1962) on the *sec*+/*sec*− heterokaryons suggests that the *sec*− phenotype is not under cytoplasmic control. Combined, these data suggest that in the process of loss of aflatoxin production, the *sec*− forms may have developed some form of vegetative incompatibility, even though they all belong to the same species. To explore this hypothesis, we are in the process of conducting protoplast fusions between representative *sec*−/*sec*− strains. If protoplast fusions in these crosses result in formation of viable heterokaryons and/or diploids, clearly, the barrier in the parasexual cycle experiments exists at the anastomosis (hyphal) level, bypassed by protoplast fusions.

More recent work on the cloning and analysis of the mating type locus in *N. crassa* (alleles A and a, Glass et al. 1988; Metzenberg and Glass 1990) and that in *Podospora*

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anserina (alleles S and s, Turcq et al. 1990) has offered a better insight into the molecular basis of vegetative incompatibility. In *N. crassa* for example, the A/a mating locus is involved in both vegetative incompatibility and in sexual reproduction (while in *A. nidulans*, the *het* genes do not influence sexual reproduction) and the sequence required for the expression of incompatibility has been localized to a single open reading frame with the putative polypeptide having similarity to the MAT alpha 1 polypeptide of *S. cerevisiae* (Glass et al. 1990). More recent work has further identified a cluster of genes surrounding the mating locus that may be involved in the heterokaryon incompatibility (Randall and Metzenberg 1998). Additional research is certain to result in characterization of these genes and will perhaps assist in discovering their corresponding homologs in other fungal systems.

8.2 Intra or Interspecies Protoplast Fusion to Study Phenomena Other than Vegetative Compatibility

In filamentous fungi, protoplast fusion experiments at the interspecies level (Anne et al. 1976) were reported around the same time as those at the intraspecies level (Anne and Peberdy 1976). Both shared similar techniques for protoplast fusions and for data analysis, using the principles of parasexual cycle. Such studies in representative fungi of academic, economic, or commercial significance are discussed below.

8.2.1 *Penicillium*

Over the span of about 15 years, several auxotrophically marked species of *Penicillium* including *P. chrysogenum*, *P. notatum*, *P. cyaneofulvum*, *P. citrinum*, *P. roqueforti*, and *P. stoloniferum* were employed in interspecies hybridizations using protoplast fusion techniques [Anne 1977; 1982a, b; Anne and Peberdy 1976; Anne et al. 1976; reviewed by Anne and Peberdy (1985)]. Since, *Penicillium* is a well-known producer of β lactam antibiotics, the main goals of these studies were two-fold: (a) to conduct basic genetic analysis, and (b) to possibly create a stable recombinant (hybrid) with improved antibiotic production. Detailed analysis of results revealed that in general, crosses involving related species like *P. chrysogenum* X *P. notatum* (Anne 1977; Anne and Peberdy 1976), and *P. cyaneofulvum* X *P. citrinum* (Anne and Eyssen 1978) formed healthy heterokaryons and upon longer incubation, gave rise to prototrophic colonies on MM that segregated under nonselective conditions. Such colonies were presumed to be diploids, but since it was not possible to say with certainty whether or not all the chromosomes from both parents were present in the potential diploid, the term "hybrid" was used (Anne and Peberdy 1985). Alternative terms like "alloploids" or "allodiploids" have also been used to describe such fusion products (Kevei and Peberdy 1985). On the other hand, if the crossing parents were from relatively less related species like *P. chrysogenum* X *P. roqueforti*(Anne et al. 1976) or *P. chrysogenum* X *P. stoloniferum* (Anne 1982a), the heterokaryons grew poorly, did not sporulate on MM, showed more aberrant morphology and under nonselective conditions, produced spores of only one of the parents, suggesting imbalanced heterokaryons and incompatibility at the chromosomal level. While it was possible to force the poorly growing heterokaryons to produce prototrophic hybrids, they often showed variable stabilities (Anne 1982a). In fact, natural or induced haploid segregants stable enough to withstand further genetic or biochemical analysis could mostly be obtained only from hybrids of related species (Anne and Peberdy 1985). Analysis showed that in general, as long as one of the parents was an antibiotic producer, the penicillin production in the hybrids and/or the segregants ranged between the two parents, providing evidence for genetic recombination (Anne 1983; Anne and Peberdy 1985). Similar tests for isoenzyme analysis (e.g., catalase) in the hybrids and their segregants showed profiles of either one parent, both parents, or novel bands, the latter indicating genetic recombination (Anne and Peberdy 1981). Based on such analyses, Anne and Peberdy (1985) discussed the potential use of *Penicillium* interspecies protoplast fusions in obtaining novel secondary metabolites, provided the limitations of the screening process and the instabilities of certain fusion products could be overcome. Since the mid-80s, there have been few reports on creation of novel penicillin producing strains. However, one relatively recent study used a slightly different approach—that of overcoming the use of auxotrophic markers (which can have a decreased effect on penicillin production, Ferenczy et al. 1987; Kikuchi 1980) by obtaining nitrate nonutilizing and acetate nonutilizing mutants of high penicillin producing strains of *P. chrysogenum* and fusing their protoplasts (Tahoun 1993). Since, the cross was at the intraspecies level, diploids were readily obtained and induced haploidization allowed recovery of stable segregants, one of which reached a penicillin G titer of 290–390% over that of the parental strains (Tahoun 1993). The antibiotic production by this novel segregant at a scale-up batch or continuous-level and its long-term stability was not discussed in this study. These are critical issues for any industrial strain, because it has been shown that in the absence of selection pressure, high penicillin producing industrial strains (usually ploidy mutants) segregate to lower producing strains by chromosomal losses (Kunkel et al. 1992). This may be one of the reasons why protoplast fusions have not always been the best choice for industrial strain improvement purposes.

8.2.2 *Aspergillus*

Results obtained from interspecies hybridizations in *Aspergillus* differ to a certain degree from those in *Penicillium* in that, even crosses of closely related species such as *A. nidulans*, *A. rugulosus*, *A. quadrilineatus*, *A. nidulans* var *echinulatus* and *A. violaceus* did not easily yield healthy heterokaryons and allodiploids (Kevei and

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Peberdy 1984). When protoplasts of taxonomically unrelated species like *A. nidulans* and *A. fumigatus* were crossed, fusion events were rare, the fusion products were highly unstable and when subcultured on CM, conidia from the fusion segregants produced only *A. nidulans* colonies (Ferenczy et al. 1977). Analysis of a small percentage of such prototrophic segregants showed that part of the *A. fumigatus* genome was responsible for overcoming the nutritional auxotrophy (Ferenczy et al. 1977), hence the term "partial aneuploid" was coined (Ferenczy 1981) to describe them. On the basis of this data, it therefore appears that vegetative incompatibility and postfusion incompatibility is more prevalent in *Aspergillus* than in *Penicillium* species. Kevei and Peberdy (1985) suggested that the use of other techniques for genetic transfers (like isolated nuclei and mitochondria) could allow scientists to induce genetic interactions at a wider level. Indeed a few years later, an efficient method for isolation of *Aspergillus* nuclei from protoplasts was reported (Vagvolgyi and Ferenczy 1991), and purified nuclei from a haploid auxotrophic *A. nidulans* strain were transferred into the protoplasts of a complementary haploid auxotroph (Vagvolgyi and Ferenczy 1992). This process, also called karyoduction, resulted in both heterokaryotic and diploid colonies with a low but reproducible frequency of nuclear uptake (Vagvolgyi and Ferenczy 1992). Production of heterokaryotic nuclear hybrids have helped overcome the barriers of incompatibility in other fungi as well as shown in a recent study where nuclei from an auxotrophic strain of *Lentinula edodes* (a pharmacologically active basidiomycete) were transferred into complementing auxotrophic protoplasts of *Coriolus versicolor* (an incompatible partner) and nuclear hybrids with mixed nonparental isozyme patterns were obtained (Kim et al. 2000b).

In another recent study, isolated nuclei of the diploid fungus *Phytophthora parasitica* were fused with protoplasts of another strain of the same species (Gu and Ko 1998; 2001). The resulting regenerated nuclear hybrids expressed increased vigor in that, they grew faster than the parental isolates. These hybrids also expressed increased fungicide resistance. Since, such a phenomenon did not occur in hybrids regenerated from mitochondrion–protoplast or protoplast–protoplast fusion products between the two strains, it indicated that the hybrid vigor was the result of the interaction between the two different kinds of nuclei, but not between the two types of mitochondria. Such increased vigor was also observed in the nuclear hybrids within two different species of *Phytophthora*, suggesting potential application of this fusion technique in creation of superior fungal strains for usage in agriculture and industry (Gu and Ko 2001).

The first studies on protoplast fusion in another industrially important organism *A. niger* (the koji fungus and well-known producer of citric acid) were reported by Kirimura et al. (1986; 1988), where they succeeded in producing fusion strains with higher productivity in solid culture methods compared to the parental strains. In another study, using protoplast fusion, a recombinant strain producing slightly higher (2.5%) beta-glucosidase production was obtained by Hoh et al. (1992). Other koji molds like *A. oryzae* and *A. sojae* have also been used in protoplast fusion experiments with some success in generating stable recombinants with desirable traits of both parents [e.g., increased protease, glutamine, or ethanol production; reviewed by Hara et al. (1992)].

8.2.3 *Claviceps*

Claviceps purpurea is another commercially important deuteromycete that is used in the production of a broad spectrum ergot alkaloids with therapeutic uses (Spalla and Marnati 1978). Both intra and interspecific level protoplast fusions have been carried out in this organism with some success in obtaining hybrids capable of either producing greater amounts of alkaloids or a combination of alkaloids [compared to the parental strains, Robbers (1984); Spalla and Marnati (1982)]. More recently, protoplast fusions between strains of *C. purpurea* carrying auxotrophic and fungicide resistance markers were conducted (Didek-Brumec et al. 1991). In this study, one parent actively produced two alkaloids and the second parent was inactive in producing a third type of alkaloid. Protoplast fusion experiments succeeded in generating prototrophic strains that produced all three types of alkaloids demonstrating recombination and activation of distinct alkaloid biosynthesis in the inactive partner (Didek-Brumec et al. 1991).

8.2.4 *Giberella*

A group in Germany first optimized the conditions for protoplast formation in *Giberella fujikuroi*, producing the horticulturally important plant growth hormones, gibberellins (Bruckner et al. 1990). Next, they isolated several auxotrophic, drug resistant, nitrate nonutilizing, and albino mutants of this species (Bermudez and Torres et al. 1992). Using contrastingly marked mutants; they then set up different combinations of crosses using the protoplast fusion technique, and made the following observations. Stable heterokaryon and diploids (induced by camphor treatment, Kirimura et al. 1988) were obtained from every cross except where both parents had drug resistance markers, and while most recombinants showed lower gibberellin titers than the parental strains, some diploids had greater productivities (Bermudez and Torres et al. 1992). Based on the poor understanding of the mechanisms of gibberellin biosynthesis especially at the regulatory level, the authors attributed their success of obtaining a higher producing diploid to a chance phenomenon, not unlike mutagenesis used in industrial strain improvement programs (Bermudez and Torres et al. 1992).

8.2.5 *Trichoderma*

Some filamentous fungi (e.g., *Trichoderma* species) have been developed as biological agents to control plant pathogens, weeds, and insects by using mutagenesis, screening of wild isolates, etc. (Stasz 1990). In *Trichoderma*,

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protoplast fusion has successfully been used to obtain improved biocontrol strains, but the genetic basis for the results could not be documented based on the usual parasexual cycle events (Harman et al. 1989; Ogawa et al. 1986). This is because unexpected data was obtained from studies at the intrastrain, intraspecies, or interspecies level. For example, even at the intrastrain level, while balanced heterokaryons were obtained, heterozygous diploids were not detected despite using inducing agents (Ogawa et al. 1986). Further, when the crossing parents were at the intraspecific or interspecific level, somatic hybrid colonies grew poorly even on nutritionally CM and upon subculturing on MM or CM, continued to undergo phenotypic changes (Stasz et al. 1988). When isozyme analysis was used, no evidence was found for heterokaryotic or heterozygous diploid intermediates, aneuploids or recombinants. It was therefore concluded that parasexual cycle could not explain the postfusion events and the authors raised the question of postfusion incompatibility occurring in this species (Harman and Stasz 1991; Stasz 1990).

Interestingly, a recent study reported the isolation of a recombinant between *Trichoderma reesei* and *A. niger* (Ai et al. 1997), demonstrating genomic compatibility and interactions between the two taxonomically diverse organisms. In addition, not only was this intergeneric recombinant stable, but its growth rate and cellulase biosynthetic activity was significantly dominant over the two parents (Ai et al. 1997). Also interestingly, other recent studies in *Trichoderma* have reported the evidence for parasexual cycle events (Furlaneto and Pizzirani-Kleiner 1992; Ogawa et al. 2000). In the latter study, protoplasts of two *T. harzianum* auxotrophic mutants (one having fungicide resistance, another, better growth pattern) were fused to obtain heterokaryons and subsequently a diploid that had both parents' desirable traits (Ogawa et al. 2000). In contrast with an earlier report (Ogawa et al. 1986), in this instance, the d-camphor treatment succeeded in inducing formation of the diploid, the ploidy of which was confirmed by various analyses. Finally, the biocontrol properties of this diploid were determined to be against *Fusarium* and *Pyricuria* (phytopathogenic fungi) species (Ogawa et al. 2000). From this recent work, it appears that some *Trichoderma* species probably participate in parasexual crossing, but depending on the compatibility issues and the nature of the genetic interaction, the products may vary in their stability and, hence may be rather difficult to study.

8.2.6 *Beauveria*

In the deuteromycete *Beauveria* (a biocontrol fungus), several protoplast fusions and parasexual recombinations have been reported (Kawamoto and Arzawa 1986; Paccola-Meirelles and Azevedo 1991; 1994; Paris 1977). More recently, an interspecies crossing in *Beauveria* resulted in obtaining a superior biocontrol strain, as follows: An entomopathogenic strain of *B. bassina* active against two insects was crossed with a strain of *B. sulfurescens* capable of producing an insecticidal toxin (Couteaudier et al. 1996). Protoplast fusions between the diauxotrophic mutants of these strains resulted in the recovery of some stable products, two of which showed increased virulence against the susceptible insects (possibly because of the combining of both parents' characteristics). Molecular RFLP analysis suggested that these were recombinants or partial diploids with portions of the genome being heterozygous. A molecular mitochondrial marker indicated homoplasmy of these hybrids, with inheritance of the mitochondria from either of the two parents. The pathogenicities and the ploidies of the hybrids remained stable after passage through the host insect, suggesting that somatic hybridization through protoplast fusion may serve as a method for genetic improvement of biocontrol mechanisms in the entomopathogenic fungi (Couteaudier et al. 1996; Viaud et al. 1998).

9 CYTOPLASMIC INFLUENCES IN PROTOPLAST FUSIONS

Since, fungi contain nucleic acid bearing organelles like mitochondria and also carry plasmids and mycoviruses, the influence of these cytoplasmic elements in the postfusion events cannot be excluded. There have been some reports on protoplast fusions to examine the transfers of mycoviruses (van Diepeningen et al. 1998; Varga et al. 1994), but most studies in this regard have focused on mitochondria, the sites of cellular respiration and energy production. In fungi, as in other eukaryotes, mitochondrial DNA (mtDNA) is generally maternally inherited and does not normally undergo recombination during sexual reproduction (Mason and Turner 1975; Mitchell and Mitchell 1952). Specific reasons for this mitochondrial behavior from the evolutionary point are not well understood but in a recent report, Kevei et al. (1997) offer possible explanations including active degradation of mitochondria of one of the parents, and selective silencing (gradual disappearance) of mitochondria of one of the parents upon zygote formation.

During asexual reproduction, however, mtDNA recombination does occur, and can be easily followed using appropriate nuclear (auxotrophy and spore color) and mitochondrial (drug resistance) markers (Rowlands and Turner 1974; 1977). According to Ferenczy (1983), nonselective transfers involve transmittance of mitochondria along with the donor nucleus via protoplast fusion with the separation of the two types of genomes occurring at a later stage. Conversely, in a selective transfer, the mitochondria are first separated and then transfused selectively into the recipient protoplasts (Ferenczy 1981). Early experiments of such nature were carried out in *S. cerevisiae* and *Schizosaccharomyces pombe* (Ferenczy and Maraz 1977; Gunge and Tamaru 1978; Luckemann et al. 1979; Maraz and Subik 1981) which have been reviewed by Ferenczy (1985). Results from these studies which included comparative analyses of normal sexual crossing vs. protoplast fusion using

several combinations of mitochondrial markers, nuclear markers, and mating types demonstrated that the transmission and recombination frequencies of mitochondrial genes were very similar in both processes (fusion vs. sexual crossing) regardless of the mating type (Ferenczy 1985). The basic experiments in yeast were soon extended to filamentous fungi demonstrating that using protoplast fusion, mitochondria can successfully be transmitted at the intra or interspecific level (Croft and Dales 1983; Croft et al. 1980).

Most recent studies have used mtDNA RFLP comparisons in addition to the nuclear and mitochondrial genetic markers to better understand the nature of mitochondrial recombination. For example, in the edible mushroom *Lentinula edodes*, mitochondrial inheritance was compared in sexual crosses vs. those induced by protoplast fusions (Fukuda et al. 1995). Since, mtDNA had been implicated earlier in influencing mycelial growth of another mushroom *Agaricua bitorquis* (Hintz et al. 1988), it was of interest to determine if the same may be true for *L. edodes*. By comparing the RFLP patterns, it was observed that all newly established dikaryons formed by sexual crossing retained the mitochondrial RFLP of one of the monokaryons, suggesting the uniparental mode of the mitochondrial inheritance (Fukuda et al. 1995). This was not an expected result, since earlier, cytological experiments have clearly shown the movement of mitochondria from one parent to another through the opening formed by hyphal anastomosis during sexual crossing (Nakai 1986). Fukuda et al. (1995) suggested that perhaps dikaryotization and cell division in *L. edodes* may occur shortly after hyphal fusion not allowing sufficient time for mitochondrial recombination to occur. In the same study, genetically recombined mitochondria were obtained using protoplast fusion, possibly, (according to the authors) because it took at least 3 days for the protoplasts to regenerate, which allowed for the genetic exchange to occur (Fukuda et al. 1995).

Other mitochondrial studies have been performed in *Aspergillus* species predominantly to study mitochondrial inheritance and to characterize the recombinations patterns in the context of strain compatibility as model systems (Coenen et al. 1996; Hamari et al. 1997; Hamer et al. 2001; Kevei et al. 1997; 2001; Kirimura et al. 1992; Toth et al. 1998). For example, using protoplast fusions, mitochondria were transferred at the intra and interspecific level among incompatible *A. niger* strains and based on the RFLP patterns, three main types of mtDNA profiles were established (Kevei et al. 1997). Since, the vegetative incompatibility in the *A. niger* strains is so strong that even protoplast fusion cannot force heterokaryon formation and interaction of the two parents' nuclei, the mitochondrial experiments were successful only under selection pressure for the oligomycin-resistant donor mtDNA and the recipient nuclear phenotype (Kevei et al. 1997). Using this directed transfer strategy, mitochondrial transfers among three mtDNA types (and their subgroups) belonging to *A. niger*, *A. tubingenesis*, and some Brazilian wild isolates were successful. However, such crosses among the more distantly related black *Aspergilli*-like *A. niger* and *A. japonicus* were unsuccessful (Kevei et al. 1997), possibly, according to the authors, due to the wide variety of naturally occurring polymorphic mtDNAs among the black *Aspergilli* (Varga et al. 1994). More recently, using similar strategy, oligomycin-resistant mitochondria were transferred to oligomycin sensitive *A. japonicus* recipients carrying appropriate nuclear markers like nutritional requirement and spore color (Kevei et al. 2001). In this study, based on the RFLP patterns, the progeny selected for oligomycin resistance with recipient nuclear phenotype had either unchanged donor mtDNA (substituted progeny) or recombinant mtDNA (recombinant progeny). The latter were found to harbor mobile elements characteristic of the recipient parents, while the former were either stable (due to compatible co-operation between the donor mitochondria and the recipient nuclei), or aconidial and unstable (due to less compatible donor mitochondrial and recipient nuclear communication, Kevei et al. 2001). Finally, the aconidial substituted progeny was found to undergo a segregation process resulting in stable, wild-type strains with a new type of mtDNA similar to that of the recipient parent (Kevei et al. 2001). In a related study, detailed analysis of a protoplast-fusion generated *A. japonicus* strain harboring a recombinant mtDNA suggested that both mobile intron losses and acquisitions played a role in formation of the rearranged mtDNA (Hamari et al. 2001). In addition, with certain parental combinations, a particular intron was lost very frequently and since intron loss is an important phenomenon in eukaryotic gene expression, this process is currently under investigation (Hamari et al. 2001).

10 CONCLUDING REMARKS

Protoplast fusions continue to play a major role in fungal research. Even DNA transformation, where protoplasts serve as recipients of exogenous vectors, is thought to be a protoplast fusion-mediated event (Vagvolgyi and Ferenczy 1992). Further, protoplasts have also been transformed by total RNA extracts, an event termed as retrotransformation (Zucchi et al. 1989). Such RNA-mediated transformations have been shown to recover gene functions lost by point mutations or deletions (Zucchi 1996). It is quite possible that retrotransformation also occurs as a result of the fusion of the recipient protoplasts.

One important feature that serves as a common thread in all protoplast fusion experiments is the genetic compatibility of the two participating parents. As seen from many of the studies discussed in the earlier sections, the greatest advantage of protoplast fusion is to overcome the vegetative incompatibility that exists at the hyphal anastomosis level and to force the formation of a heterokaryon. Once this is accomplished, formation of diploids, recovery of recombinant segregants, etc., will depend on other factors such as interactions between the two types of nuclei, influence of cytoplasmic elements, and the level of compatibility/tolerance among all the heterogenous DNA components existing in the fusion product. However, protoplast fusions

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cannot always force the formation of a stable, balanced heterokaryon because the incompatibility may be so extreme that it is not possible for the two types of nuclei to coexist. In such cases, nuclear DNA of only one of the parents (sometimes with portions of the other parents' genome) is likely to remain in the fusion product, determining the outcome of the parasexual analysis. In fact, compatibility issues are known to affect production of industrially important secondary metabolites (Anne 1983). Thus, due to the issues of strain stability and the complexity of genetic regulatory processes of secondary metabolic pathways, increased use of protoplast fusions for generation of novel industrial strains is not very probable. However, intra and interspecies protoplast fusions will very likely be widely used in relation to taxonomic (e.g., vegetative incompatibility), evolutionary (e.g., mitochondrial inheritance), and agricultural (e.g., generating superior biocontrol strains) processes.

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3

Metabolic Pathway Regulation

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1 INTRODUCTION

Research efforts in the past three decades have revealed the complex mechanisms employed by fungi to control gene activity. In their natural habitat, fungi are capable of using a variety of compounds as nitrogen and/or carbon sources. However, not all nitrogen and/or carbon sources support growth and development equally. This chapter will focus primarily on the regulatory mechanisms governing nitrogen and carbon metabolism, particularly on utilization of alternative nitrogen and carbon sources, in the model filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. Recent progress in the characterization of global factors affecting nitrogen and carbon metabolism, and pathway-specific metabolic gene regulation will be emphasized. Global regulators act on a wide range of unrelated activities involved in utilization of an array of different compounds, whereas pathway-specific regulators usually act specifically within a single or several related pathways to mediate coordinated expression of activities required for utilization of related compounds. A number of authoritative reviews of early aspects of regulation of nitrogen and carbon metabolism have been published (Caddick et al. 1994; Ebbole 1998; Kelly 1994; Felenbok and Kelly 1996; Marzluf 1997; Ruijter and Visser 1997; Scazzocchio et al. 1995).

2 REGULATION OF NITROGEN METABOLISM

In order to use a nitrogenous compound as a nitrogen source, the compound has to be converted to glutamate and glutamine, which serve as nitrogen donors for the synthesis of all other nitrogen-containing compounds in the cell. Both glutamate and glutamine can be synthesized using ammonium as the amino group donor. Therefore, ammonium, glutamate, and glutamine are the preferred nitrogen sources. In the absence of these favored compounds, secondary nitrogen sources such as nitrate, amides, purines, amino acids, and proteins are used. The phenomenon of preferential utilization of favored nitrogen sources is known as nitrogen metabolite repression (NMR). This mechanism enables fungi to efficiently manage the available nitrogen resources, and only under conditions of nitrogen limitation will alternative pathways responsible for the utilization of secondary nitrogen compounds be activated. Selection of the best nitrogen source must include a sensing mechanism. Glutamine is a key effector of nitrogen metabolite repression but the intracellular concentration of glutamine appears not to be the sole determinant (Morozov et al. 2001). Ammonium, in combination with alpha-ketoglutarate, can be converted to glutamate through the action of NADP-dependent glutamate dehydrogenase or the combined action of glutamine synthetase and glutamate synthase (Macheda et al. 1999). Glutamate and ammonium also act to signal nitrogen sufficiency independently from the glutamine signal (Margelis et al. 2001).

2.1 Global Positive-Acting Nitrogen Pathway Regulators

The utilization of secondary nitrogen sources requires the relief of nitrogen metabolite repression to allow *de novo*

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synthesis of specific permeases and catabolic enzymes. Relief of repression (derepression) is mediated by global positive-acting regulatory proteins. Such regulators are encoded by *areA* of *A. nidulans* (Kudla et al. 1990), *A. oryzae* (Christensen et al. 1998), *A. niger* (MacCabe et al. 1998) *A. parasiticus* (Chang et al. 2000), and *Gibberella fujikuroi* (Tudzynski et al. 1999), *nit-2* of *N. crassa* (Fu and Marzluf 1990a,b), *nre* of *Penicillium chrysogenum* (Haas et al. 1995; Haas and Marzluf 1995) and *NUT1* of *Magnaporthe grisea* (Froeliger and Carpenter 1996). All these regulators are members of the Cys-X_2-Cys-X_{17}-Cys-X_2-Cys zinc finger GATA-binding transcription factor family, and some have been shown to bind DNA with the consensus sequence HGATAR (Fu and Marzluf 1990b; Ravagnani et al. 1997). Various mutations that affect AreA activity have been extensively characterized (Arst and Cove 1973; Caddick et al. 1994; Langdon et al. 1995; Stankovich et al. 1993; Wilson and Arst 1998). Loss-of-function alleles confer inability to utilize nitrogen sources other than ammonium and are commonly associated with changes in the DNA-binding domain. The *areA102* mutation results in the substitution of the highly conserved leucine within the zinc finger for a valine, and causes a complex phenotype, including increased *amdS* and reduced *uapA* expression, due to altered GATA-site specificity (Hynes 1975; Kudla et al. 1990; Ravagnani et al. 1997).

In *A. nidulans*, AreA activity is modulated to facilitate expression of genes subject to nitrogen metabolite repression only under conditions of nitrogen limitation (Platt et al. 1996a). This modulation is achieved through control of *areA* transcription, altered stability of the *areA* mRNA, and interactions of AreA with other proteins (Langdon et al. 1995; Platt et al. 1996a,b; Small et al. 1999). The *areA* transcript exists at extremely low levels in nitrogen repressing conditions but is highly expressed in derepressing conditions. Transcripts of three sizes, approximately 3.9, 3.6, and 3.2 kb, are produced and appear functionally redundant. Synthesis of the 3.2-kb transcript is subject to autogenous regulation (Langdon et al. 1995). The turnover rate of the *areA* transcript depends on nitrogen status. The wild-type *areA* transcript has a half-life of 40 min in derepressing conditions compared to 7 min in repressing conditions (Platt et al. 1996a). Deletion of a 218 bp region in the 3′ untranslated region (UTR) of *areA* results in partial derepression of *areA*-regulated activities (Platt et al. 1996a). This 218 bp sequence is sufficient to confer nitrogen-regulated transcript degradation on a reporter gene (Morozov et al. 2000). Deletion of a 58 nucleotide region within this sequence confers a half-life of about 25 min for *areA* mRNA in repressing conditions and a parallel derepressed phenotype (Platt et al. 1996a). This sequence is required for accelerated shortening of the poly-A tail, which in turn leads to the rapid degradation of the *areA* transcript under repressing conditions (Morozov et al. 2000; Morozov et al. 2001). Intriguingly, the *N. crassa nit-2* mRNA and NIT2 protein are easily detectable regardless of the nitrogen source, and the *nit2* transcript is quite stable (Fu and Marzluf 1987). Nonetheless, the levels of the *nit-2* transcript and the NIT2 protein increase three to four-fold when a secondary nitrogen compound is the sole nitrogen source (Tao and Marzluf 1999).

The activity of the AreA protein, in addition to *areA* transcript levels, is modulated. The 12 amino acid residues at the carboxyl terminus, EWEWLTMSL, are necessary for modulation of AreA function. Noticeably, 9 out of the 12 amino acid residues at the carboxyl termini of AreA homologs from *Aspergillus* species (*nidulans*, *niger*, *oryzae*, and *parasiticus*), *P. chyrosogenum*, *N. crassa*, and *G. fujikuroi* are identical (*M. grisea* has a conserved amino acid substitution, EWDWLTMSL), strongly suggesting functional conservation of this region. Deletions within the carboxyl terminus of AreA result in derepression to levels similar to those caused by 3′ UTR deletions in *areA* (Platt et al. 1996b). As the effects of mutations in these two regions are additive, the carboxyl terminus and 3′UTR define independent control mechanisms for regulation of *areA* activity.

2.2 Repressors Involved in Nitrogen Metabolite Repression

The nitrogen control circuit for the utilization of secondary nitrogenous compounds involves negative-acting regulatory proteins. Their function is to antagonize the action of the corresponding activators. The *N. crassa* negative regulatory protein NMR, encoded by the *nmr* gene (for *n*itrogen *m*etabolite *r*epression) (Fu et al. 1988), modulates the activity of NIT2 and thereby alters expression of many nitrogen metabolic genes (Marzluf 1997). The *nmr* gene is constitutively expressed at a very low level. Up to 16% of the carboxyl terminus of NMR is not necessary for function (Young et al. 1990). The NMR repressor is able to interact with two distinct regions of the NIT2 activator: a short alpha-helical motif within the NIT2 DNA-binding domain and an alpha-helical motif at the carboxyl terminus (Xiao et al. 1995). These interactions probably block the functional domains of NIT2, thus either interfering with binding of NIT2 to GATA sites, shielding the nuclear localization sequence (NLS) located in the basic region, or making regions of NIT2 inaccessible to coactivators. Various mutations in *nmr* resulted in derepression of many activities in the presence of ammonium or glutamine suggesting that the overall conformation of NMR is critical for its activity (Tomsett et al. 1981). Disruption of *A. nidulans nmrA*, the homolog of *nmr*, resulted in a partially derepressed phenotype similar to those of *areA* mutants with alterations in the DNA binding domain and the carboxyl terminus (Andrianopoulos et al. 1998). The predicted NmrA protein contains five regions highly conserved with *N. crassa* NMR. It is likely that protein–protein interactions occur between NmrA and the AreA DNA-binding domain and carboxy-terminal 12 amino acid region. Most recently, the crystal structure of NmrA has been resolved (Nichols et al. 2001) and, unexpectedly, NmrA

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displays structural similarity to the short-chain dehydrogenase/reductase family (Stammers et al. 2001).

2.3 Other Regulatory Genes Related to Nitrogen Metabolism

2.3.1 *Aspergillus nidulans* TamA is a Coactivator of AreA

Mutations in *A. nidulans tamA* result in simultaneous resistance to toxic analogues such as *t*hiourea, *a*spartate hydroxamate and *m*ethylammonium (Kinghorn and Pateman 1975). These analogues are substrates of enzymes or permeases, which are regulated by nitrogen metabolite repression. The *tamA* mutants were referred to as "partially repressed" in terms of nitrogen metabolite repression, showing reduced growth on ammonium and reduced levels of a variety of nitrogen metabolic enzymes (Kinghorn 1999). The *tamA* gene has been cloned by complementation (Davis et al. 1996). The predicted TamA protein contains a Zn(II)2Cys6 zinc binuclear cluster DNA-binding motif, similar to other known pathway-specific regulatory proteins found in fungi (see Section 4.1). However, the zinc cluster motif is not required for function (Davis et al. 1996). Domain-swap experiments show that residues 153–651 of TamA are sufficient for function, and TamA apparently acts as a transcriptional coactivator (Small et al. 1999). Random amino acid sequence changes and internal deletions abolish the function of TamA, which suggest that overall confirmation is critical for its activity (Small et al. 2001). TamA interacts with the carboxy-terminal region of AreA (Small et al. 1999). Interestingly, this is the same region in AreA predicted to interact with the negative-activing NmrA protein of *A. nidulans* (Platt et al. 1996b). Thus, TamA may function to displace the negative-acting NmrA protein under derepressing conditions (Small et al. 2001). Consistent with its role as a transcriptional coactivator, TamA contains sequences showing similarity to putative NLSs (Small et al. 2001). Furthermore, a GFP-TamA fusion protein was found predominantly in the nucleus regardless of the presence or absence of ammonium, and *areA* was not required for this distribution (Small et al. 2001). TamA is highly conserved, with homologs in *A. oryzae* (Small et al. 2001) and in the *N. crassa* genome sequence (R.B.T., unpublished observations), suggesting that TamA homologs function similarly in other fungi.

2.3.2 Negative-Acting DNA-Binding Factors: *Penicillium chrysogenum* NreB and *Aspergillus nidulans* AreB

The *nreB* gene from *P. chrysogenum* encodes a GATA zinc finger DNA-binding factor with similarity to Dal80p/Uga43p and Gzf3p/Nil1p, both repressors of nitrogen metabolism in *Saccharomyces cerevisiae* (Haas et al. 1997). NreB also contains a putative basic leucine zipper domain. Overexpression of *nreB* leads to repression of nitrogen catabolic genes. Two *nreB* transcripts, 1.5 and 1.8 kb in length, are produced, and the steady state level of these transcripts is subject to nitrogen metabolite repression (Haas et al. 1997). Antisense *nre* (the *areA* homolog) simultaneously represses the expression of *nre* and *nreB*, which further suggests that *nreB* is under *nre* regulation (Zadra et al. 2000).

The *A. nidulans nreB* homolog, named *areB*, was identified by screening for suppressors of *areA* loss-of-function mutations (Conlon et al. 2001). The *areB* gene is predicted to encode at least three distinct proteins, which arise from different promoters, differential splicing and AUG and non-AUG translation start codons. All the putative products include a GATA zinc finger domain and a leucine zipper motif similar to the one identified in NreB (Conlon et al. 2001). Three classes of mutations in *areB* have been identified; (a) loss-of-function mutations resulting from termination of translation within or before the GATA domain, (b) mutations resulting in proteins retaining the GATA domain but truncated either at or upstream of the leucine zipper, and (c) gain-of-function rearrangements fusing novel gene sequences to *areB* to produce chimeric polypeptides. The novel fusion genes observed in the gain-of-function mutants convert the putative negative-acting transcription factor into an activator that can partially replace *areA*.

2.3.3 The *meaB* Gene Of *Aspergillus nidulans*

Methylammonium, a toxic substance, can induce nitrogen metabolite repression in wild type *A. nidulans*. Methylammonium toxicity can be reversed by ammonium and by mutations that increase the conversion of this substance to ammonium. Mutations at the *meaB* locus result in derepression of a number of activities subject to nitrogen metabolite repression (Arst and Bailey 1980). The *meaB* gene has been isolated (Polley and Caddick 1996). The expression of *meaB* is independent of nitrogen metabolite repression. In addition, *meaB* mutants showed no changes in the level of the transcript produced, which suggests that *meaB* is not autoregulated. Pfam (protein families database of alignments and hidden Markov models) common domain search reveals weak support for MeaB as a member of the basic leucine zipper (bZIP) transcription factor family. However, like bZIP proteins, MeaB contains a putative basic DNA-binding domain, an invariant asparagine, and a 6-amino acid connector (McKnight 1991) positioned N-terminal to a putative leucine zipper. The predicted MeaB leucine zipper-like region is Leu-X6-Asn-X6-Leu-X6-Ala-X6-Leu-X6-Leu compared to the consensus sequence Leu-X6-Leu-X6-Leu-X6-Leu-X6-Leu. Asparagine and alanine are found at leucine positions 2 and 4, respectively, but are not predicted to interrupt the extensive alpha-helical structure. Substitutions of a leucine residue(s) for other residues in various positions that do not disrupt function have been reported for bZIP proteins (McKnight 1991; Peters et al. 2001). The Leu to Ala substitution also is present in the putative leucine zipper of AreB and NreB. Leucine zippers are commonly involved in

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protein dimerization. Whether MeaB engages in specific protein–protein interactions with the global nitrogen regulatory protein, AreA, thereby eliciting important regulatory responses remains to be studied.

3 REGULATION OF CARBON METABOLISM

Fungi encounter a wide range of potential carbon sources in the environment and have evolved a large number of metabolic pathways to allow utilization of many carbon compounds. Some carbon sources are more complex, and consequently less energetically efficient to utilize, than others. Metabolic pathways for carbon source utilization are highly regulated to allow the preferential utilization of energetically favorable compounds such as glucose.

3.1 Mutations Affecting Carbon Catabolite Repression (CCR) In *Aspergillus nidulans*

Carbon catabolite repression (CCR) is the repression in the presence of a preferred carbon source (e.g., glucose) of genes required for utilization of nonpreferred carbon sources (e.g., ethanol, starch, acetate, and quinate). CCR acts on many metabolic pathways that are required for utilization of carbon sources. Genetic screens for mutations affecting CCR in *A. nidulans* exploited the regulation of utilization of compounds that serve as both carbon and nitrogen sources, such as proline or acetamide [reviewed in Felenbok and Kelly (1996); Scazzocchio et al. (1995)]. Relief of either NMR or CCR is sufficient for expression of the genes required for utilization of these compounds. *areA* loss-of-function mutations confer inability to grow on compounds that serve as both carbon and nitrogen sources in the presence of glucose. Mutations affecting CCR were selected as extragenic suppressors of *areA* loss-of-function mutations on such compounds in the presence of glucose and mapped to three genes: *creA, creB,* and *creC* (Arst and Cove 1973; Kelly 1980; Hynes and Kelly 1977). *creA* mutants have also been isolated using other selection strategies (Bailey and Arst 1975; Shroff et al. 1997). Mutations in *creA* cause derepression of a wide range of activities, such as those required for utilization of ethanol, acetate, and starch, which would normally be repressed in the presence of repressing carbon sources.

3.2 Global Regulators of Carbon Metabolism

3.2.1 CreA, a DNA-Binding Transcriptional Repressor

The *A. nidulans creA* gene encodes a 416 residue protein containing two Cys2His2 zinc finger DNA-binding motifs (residues 66–116) and an alanine-rich region (residues 131–132) (Dowzer and Kelly 1989; Arst et al. 1990; Dowzer and Kelly 1991). CreA also contains a putative ubiquitination consensus sequence (residues 258–267, HEDEDSYASH) (Strauss et al. 1999). CreA shows similarity through the zinc finger region to Mig1p, the major repressor of carbon catabolite repressed genes in *S. cerevisiae* (Nehlin and Ronne 1990) (see Section 3.2.2). CreA contains neither obvious NLS nor sequences with strong similarity to the NLS of Mig1p (De Vit et al. 1997). However, the predicted size of CreA is approximately 45 kDa and thus CreA may be sufficiently small to diffuse through the nuclear pore into the nucleus without active transport.

A number of studies have shown that a CreA fusion protein binds to specific DNA binding sites upstream of many genes that are subject to CCR with the consensus sequence 5′-[G/C][C/T]GG[G/A]G-3′ (Cubero and Scazzocchio 1994; Dzikowska et al. 1999; Espeso and Penalva 1994; Ho et al. 1995; Kulmburg et al. 1993; Mathieu and Felenbok 1994; Orejas et al. 1999). *In vivo* studies have demonstrated that at least some *in vitro* CreA DNA-binding sites and sequences conforming to the consensus binding site are functional (Cubero and Scazzocchio 1994; Mathieu et al. 2000; Orejas et al. 1999; Panozzo et al. 1998).

A comprehensive molecular analysis of *creA* mutant alleles showed that seven alleles carried missense mutations, and 12 alleles contained nonsense or frameshift mutations resulting in truncated CreA proteins (Shroff et al. 1996; Shroff et al. 1997). All of the missense mutations, including the extreme *creA306* allele, mapped to the DNA-binding domain, suggesting that they affect CreA DNA-binding activity. Truncation of the C-terminal 80 residues results in derepression of several activities subject to CCR, suggesting that the CreA C-terminus is required for mediating repression (Shroff et al. 1997). The *creA303* mutation truncates the CreA protein within the N-terminal zinc finger domain at residue 68, and, consistent with the extreme *creA303* phenotype, is therefore expected to be a null mutant (Shroff et al. 1997). Thus, *creA* null alleles are viable (Shroff et al. 1997). In contrast, initial deletion experiments only obtained gene replacement of *creA* in a diploid or a heterokaryon and deletion of *creA* appeared lethal (Dowzer and Kelly 1991). As haploid *creA* deletion conidia germinated but then arrested in growth, the *creA* deletion phenotype was termed "leaky-lethal" (Dowzer and Kelly 1991). Subsequently, precise gene replacement of *creA* demonstrated that a *creA* deletion mutant is viable and the leaky-lethal phenotype was due to deletion of sequences 3′ to the *creA* gene (Shroff et al. 1997).

Northern analysis revealed that *creA* is expressed in both carbon repressing and derepressing conditions, with expression levels varying according to the carbon source (Strauss et al. 1999; Shroff et al. 1996). *creA* mRNA levels were higher in derepressing than repressing conditions (Strauss et al. 1999; Shroff et al. 1996). Furthermore, in loss-of-function *creA* mutants the *creA* transcript levels in repressing conditions were increased compared with wild type to levels similar to those found in derepressing conditions suggesting that *creA* may be autoregulated (Shroff

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et al. 1996). As the 5′ DNA sequence of *creA* contains consensus CreA DNA-binding sites (Dowzer and Kelly 1991; Shroff et al. 1996), Shroff et al. (1996) suggested that *creA* autoregulation may be direct. *In vitro* mutation of two of the closely spaced CreA consensus DNA-binding sites in the *creA* 5′ regulatory region resulted in derepression of *creA* under repressing conditions when introduced into *A. nidulans* (Strauss et al. 1999). The CreA fusion protein binds oligonucleotides containing these two DNA-binding sites *in vitro* and mutation of these DNA-binding sites virtually abolished DNA-binding (Strauss et al. 1999). The levels of *in vitro* binding to these oligonucleotides by a protein that may correspond to CreA in *A. nidulans* cell-free extracts decreased in derepressing compared with repressing conditions (Strauss et al. 1999). Thus in derepressing conditions, although *creA* mRNA levels are increased, CreA protein may be subject to post-transcriptional modification resulting in degradation or modulation of DNA-binding activity.

3.2.2 Phosphorylation and Nuclear Localization Regulate Repression Function of CreA Homologs

Homologs of *creA* have been isolated from a number of filamentous fungi including *A. niger* (Drysdale et al. 1993), *G. fujikuroi* (Tudzynski et al. 2000), *Botrytis cinerea* (Tudzynski et al. 2000), *Trichoderma reesei* (Ilmen et al. 1996; Strauss et al. 1995), *Trichoderma harzianum* (Ilmen et al. 1996), *N. crassa* (de la Serna et al. 1999), *Metarhizium anisopliae* (Screen et al. 1997) and *Sclerotinia sclerotiorum* (Vautard et al. 1999). Protein sequence alignments of CreA homologs reveal strong conservation through the DNA-binding domain and several additional regions of weak conservation (Drysdale et al. 1993; Ilmen et al. 1996). One region shows similarity to RGR1, which is involved in CCR in *S. cerevisiae* (Drysdale et al. 1993; Sakai et al. 1988). However, domain-swap experiments showed that RGR1 cannot substitute for this region in *A. nidulans* CreA (Shroff et al. 1996). These *creA* homologs appear to function similarly in CCR to repress transcription of regulated genes in the presence of a repressing carbon source. Some differences are apparent amongst these CreA homologs. *A. nidulans creA*, *T. harzianum cre1* and *S. sclerotiorum cre1* mRNA levels are regulated according to carbon source. However, unlike *A. nidulans creA* and *T. harzianum cre1*, where transcript levels are higher in derepressing than repressing conditions, *S. sclerotiorum cre1* transcript levels are higher in repressing than derepressing conditions (Vautard et al. 1999). The *S. sclerotiorum* Cre1 protein is observed under derepressing conditions, though at reduced levels compared with repressing conditions (Vautard-Mey et al. 1999).

Subcellular fractionation studies indicated that *S. sclerotiorum* Cre1 was both nuclear and cytosolic under repressing conditions but largely excluded from the nucleus under carbon derepressing conditions, implicating regulated subcellular localization as a mechanism for modulating Cre1 activity (Vautard-Mey et al. 1999). Furthermore, in the same study, Cre1 showed regulated nuclear localization dependent on carbon source when expressed as a GFP fusion in *A. nidulans*. Thus, the machinery for regulation of Cre1 subcellular localization is conserved in *A. nidulans*. The repression activity, but not subcellular localization, of *S. sclerotiorum* Cre1 is modulated by phosphorylation when expressed in *A. nidulans* (Vautard-Mey and Fevre 2000). It is unknown if *A. nidulans* CreA is regulated in response to carbon source by phosphorylation or subcellular localization.

In *S. cerevisiae*, CCR is effected predominantly by the DNA-binding transcriptional repressor Mig1p, a Cys2His2 DNA-binding protein that shows similarity through the DNA-binding domain with CreA (Nehlin and Ronne 1990). Mig2p and Mig3p are paralogs of Mig1p and appear to be at least partially redundant in function with Mig1p (Lutfiyya and Johnston 1996; Lutfiyya et al. 1998). The function of Mig1p has been well characterized (Carlson 1998; Carlson 1999; Gancedo 1998; Johnston 1999). Mig1p acts by binding to a specific sequence similar to that bound by CreA in the upstream regulatory regions of regulated genes. Mig1p mediates glucose repression by recruitment of the Tup1p pleiotropic transcriptional repressor to glucose repressed promoters (Treitel and Carlson 1995). Tup1p is a seven WD40 repeat protein, which acts as a complex with the tetratricopeptide (TPR) protein Ssn6p (Komachi et al. 1994; Treitel and Carlson 1995; Williams and Trumbly 1990). Tup1p confers repression by interaction with RNA polymerase II holoenzyme components and, alteration of the chromatin structure at regulated promoters through interactions with histone deacetylases (HDAC) and histones H3 and H4 (Edmondson et al. 1996; Wu et al. 2001; Zaman et al. 2001). Mig1p activity is regulated by Snf1p kinase-dependent phosphorylation (De Vit et al. 1997; Ostling and Ronne 1998; Smith et al. 1999; Treitel et al. 1998) and subcellular localization (De Vit et al. 1997; DeVit and Johnston 1999). Under carbon limiting conditions Mig1p is phosphorylated and found in the cytoplasm whereas in the presence of glucose Mig1p is not phosphorylated and accumulates in the nucleus.

3.2.3 *Aspergillus nidulans* CreB and CreC Are Involved in Deubiquitination and CCR

The *creB* and *creC* genes were identified in the same manner as *creA* in screens for suppressors of *areA* loss-of-function mutations on compounds that serve as both sole nitrogen and sole carbon sources in the presence of glucose (Hynes and Kelly 1977). *creB* was previously identified in a screen for molybdate resistance as *molB* (Arst et al. 1970). The *creB* and *creC* mutants share the same range of pleiotropic phenotypes, including derepression of a subset of *creA*-repressed activities in the presence of glucose, poorer growth on a range of sole carbon sources (e.g., 0.5% quinate and 50 mM proline) and elevated levels of some enzymes, such as extracellular proteases, in both repressing and derepressing conditions (Arst 1981; Hynes and Kelly 1977; Kelly and Hynes 1977). Furthermore, the *creB* and *creC* phenotypes are not additive,

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suggesting that *creB* and *creC* act in the same complex or pathway and that each of these gene products is required for the activity of the other and that is its only function (Arst 1981; Lockington and Kelly 2001).

The *creB* gene was cloned by complementation and sequence analysis revealed an encoded protein of 767 amino acids containing ubiquitin carboxy-terminal hydrolase family 1 and 2 signature sequences and showing similarity with the UBP family of deubiquitinating enzymes (Lockington and Kelly 2001). CreB also contains four PEST sequences (Lockington and Kelly 2002). The determination of sequence changes in *creB* mutants revealed two alleles with missense mutations in residues absolutely conserved among deubiquitinating enzymes and two alleles containing mutations leading to a truncated CreB protein (Lockington and Kelly 2001). One of these truncation mutations results in expression of only the first 12 residues of CreB and is therefore essentially a null allele. CreB was shown to have deubiquitinating activity when expressed in a bacterial system (Lockington and Kelly 2001).

The *creC* gene was cloned by complementation and sequence analysis showed that *creC* encodes a protein of 630 amino acids containing an N-terminal proline-rich region (residues 43–123), a putative NLS (residues 168–174), and five WD40 repeat units (residues 286–595) (Todd et al. 2000). CreC shows weak sequence similarity with the *S. cerevisiae* glucose transcriptional repressor Tup1p (see Section 3.2.3). Determination of the molecular nature of mutations in three *creC* alleles and deletion analysis revealed that the C-terminal 32 residues are dispensable and residues 588-598 within the most C-terminal WD40 repeat are required for CreC function (Todd et al. 2000). Gene replacement of the *creC* gene resulted in a phenotype identical to that of the *creC* truncation mutants (Lockington and Kelly 2001). CreB and CreC are both highly conserved, each with homologs in higher organisms, but neither protein has close sequence homologs in *S. cerevisiae* (Lockington and Kelly 2001; Todd et al. 2000).

The identical range of phenotypic effects and non-additivity of mutations in *creB* and *creC* has led to the suggestion that CreB and CreC act together in a complex to deubiquitinate target proteins with both proteins required for activity (Lockington and Kelly 2001). This hypothesis is supported by co-immunoprecipitation studies, which show physical interaction of CreB and CreC in *A. nidulans* protein extracts (Lockington and Kelly 2002). Overexpression analysis showed that when overexpressed *creB* can function in a *creC* deletion mutant whereas overexpressed *creC* does not function in a *creB*$^{-}$ mutant (Lockington and Kelly 2002). Therefore it appears that CreB acts downstream of CreC and encodes the active component of the CreB–CreC complex whereas CreC acts to stabilize the CreB deubiquitinating enzyme. Furthermore, the deubiquitinating activity of CreB implicates a deubiquitination mechanism in CCR in *A. nidulans*. In *S. cerevisiae*, Skp1p-Grr1p mediated ubiquitination has been implicated in CCR as introduction of a heterologous *SKP1* homolog leads to destabilization of the F-box protein Grr1p and inactivation of Mig1p (Li and Johnston 1997; Schouten et al. 2000).

The CreA protein contains a ubiquitination motif (Strauss et al. 1999) and therefore is likely to be ubiquitinated and a target for CreB-dependent deubiquitination. A genetic association has been established between *creA* and *creB*—the *creB9730* mutation was selected as a suppressor of the most extreme *creA306* allele (Lockington and Kelly 2001). Lockington and Kelly (2002) have proposed a scheme in which CreB functions as both a free entity and in a complex with CreC. Free CreB acts under carbon catabolite repressing conditions to deubiquitinate CreA thereby preventing targeted proteasomal degradation of CreA. Deubiquitinated CreA would bind to carbon catabolite repressible promoters and repress transcription. Under carbon catabolite derepressing conditions free CreB would be degraded and CreA would be ubiquitinated and targeted for degradation via the proteasome, allowing derepression of transcription. The CreC-bound CreB is required for expression of some pathway-specific genes under both repressing and derepressing conditions.

3.2.4 Filamentous Fungal Homologs of Tup1p Play Little or no Role in CCR

In *S. cerevisiae*, CCR is mediated by Tup1p, a pleiotropic transcriptional repressor (see Section 3.2.2). Sequence homologs of *S. cerevisiae* Tup1p have been identified in filamentous fungi. The *A. nidulans rcoA* gene was cloned by homology to *TUP1* (Hicks et al. 2001). Analysis of the *rcoA* deletion phenotype indicated that *rcoA* is involved in regulation of morphogenesis and sterigmatocytin biosynthesis but plays little or no role in CCR (Hicks et al. 2001; RB Todd, unpublished data). Similarly, the *Penicillium marneffei* Tup1p homolog TupA is involved in regulation of developmental programs and plays only a minor role in CCR (Todd et al. 2003; RB Todd, MJ Hynes, and A Andrianopoulos, submitted). In *N. crassa* the Tup1p homolog RCO1 also is involved in regulation of development and repression of several genes under carbon limiting but not carbon repressing conditions (Yamashiro et al. 1996; Ebbole 1998; Lee and Ebbole 1998). In *A. nidulans*, *P. marneffei*, and *N. crassa* it appears that RcoA, TupA, and RCO1, respectively, regulate development, but it is possible that these Tup1p homologs regulate development in response to carbon status.

3.2.5 How Does CreA Effect Repression?

It is clear that RcoA does not play a major role in mediating CreA-dependent repression of carbon catabolic genes. Moreover, CreB and CreC appear to function in repression in a deubiquitination mediated process distinct from the Tup1p repression mechanism. Thus the mechanisms of CCR are not conserved between the filamentous fungi and *S. cerevisiae*. A key question remaining is how CreA effects repression at promoters of carbon catabolite repressed genes.

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A protein equivalent in function to Tup1p, which mediates repression by interaction with the transcriptional machinery and chromatin reorganization, may exist in *A. nidulans*. Alternatively, CreA itself may actively repress transcription by direct interaction with the transcriptional apparatus or directly influencing chromatin structure.

3.2.6 Other Global Regulators of CCR

The *creD* gene was identified as *cre-34*, a suppressor of the toxic effects in a *creC27* mutant of fluoroacetamide in the presence of glucose (Kelly and Hynes 1977). The *creD* mutation suppresses some but not all of the pleiotropic phenotypes of both the *creB15* and *creC27* mutants. The role of *creD* in CCR remains to be elucidated. The signals for CCR are not understood in filamentous fungi. In *N. crassa rco-3* encodes a low affinity glucose transporter which may function as a glucose sensor to signal extracellular glucose concentration (Madi et al. 1997). Thus, RCO3 may be a key element in triggering CCR. In *S. cerevisiae* hexokinases may be involved in triggering CCR [reviewed in Ruijter and Visser (1997)]. The *A. nidulans frA* gene, which encodes a hexokinase, does not appear to be involved in CCR of a number of enzymes analyzed (Ruijter et al. 1996). However, the *creA30* mutation was selected as a suppressor of *frA1* (Arst et al. 1990). Furthermore, the *xprF* gene has been recently shown to encode a hexokinaselike protein involved in the regulation of extracellular proteases (Katz et al. 2000). Katz et al. (2000) also have identified *xprG* as a suppressor of *xprF*. The role of these genes in CCR remains to be determined.

4 PATHWAY-SPECIFIC REGULATORS OF NITROGEN AND CARBON METABOLISM

In some cases, the global regulators are the only transcription factors that control gene expression, with no additional pathway-specific regulatory factors required for expression [e.g., *fmdS* (Fraser et al. 2001)]. At many promoters the globally acting protein(s) do not function alone, but require a pathway-specific regulatory protein to mediate induction by a substrate or an intermediate of the pathway. Dissection of the *amdS* and *prnB-D* promoter regulatory regions has established that the pathway-specific activators act independently of global regulators, as induction and global controls are separable (Davis et al. 1993; Scazzocchio 1992).

4.1 Domain Structure of Pathway-Specific Regulators

Pathway-specific regulators generally comprise a DNA-binding domain for interaction with specific DNA-binding sites, an effector domain for response to environmental cues, and a transcriptional activation domain that mediates transcription. In fungi the Zn(II)2Cys6 zinc binuclear cluster has been extensively used as the DNA-binding domain in pathway-specific regulators (Todd and Andrianopoulos 1997), although other DNA-binding domains, such as the Cys2His2 zinc finger, are known [e.g., AmdA, AmdX (Andrianopoulos et al. 1997; Murphy et al. 1997)]. The structure of the DNA-binding domains, recognition sites, and DNA-binding specificity of various Zn(II)2Cys6 pathway-specific regulatory proteins have been well defined [e.g., NirA, NIT4, UaY, FacB (Fu et al. 1995; Punt et al. 1995; Suarez et al. 1995; Todd et al. 1998)] and extensively reviewed (Todd and Andrianopoulos 1997). The domain structure of Zn(II)2Cys6 regulators has been established by experiments swapping DNA-binding and transcriptional activation domains between members of this class, e.g., AmdR and FacB (Parsons et al. 1992; Todd et al. 1997). Most Zn(II)2Cys6 proteins appear to bind DNA as a dimer, e.g., NirA, NIT4 (Fu et al. 1995; Strauss et al. 1998), although AlcR binds as a monomer (Nikolaev et al. 1999). Dimerization is mediated by a coiled-coil motif C-terminal to the DNA-binding domain and these domains are spaced by a linker region. Activation domains are not conserved, indicating that there may be numerous mechanisms for recruitment of the general transcriptional machinery.

4.2 Action of Pathway-Specific Regulators

There appears to be no common mechanism by which pathway-specific activators respond to signals to modulate their activity and confer changes in gene expression. Some pathway-specific regulators require inducer for DNA-binding [e.g., UaY, NirA, PrnA (Gomez et al. 2002; Oestreicher et al. 1997; Narendja et al. 2002)] whereas others may bind to promoters in the absence of added inducer [e.g., AmdR (van Heeswijck and Hynes 1991)]. Some regulators [e.g., PrnA (Gomez et al. 2002)] are constitutively localized to the nucleus whereas others show regulated nuclear localization [e.g., FacB (A. Andrianopoulos, pers. comm.)]. The activity of the QutA activator appears to be modulated by binding of the QutR repressor (Levett et al. 2000).

Many regulatory genes are expressed constitutively, e.g., *nit-4, nirA, prnA*(Fu et al. 1989; Cazelle et al. 1998; Burger et al. 1991). In contrast, expression of others is repressed by CreA and induced by an autoregulatory mechanism [e.g., *alcR*, *qutA*, *qa-1F* (Patel and Giles 1985; Levesley et al. 1996; Lockington et al. 1987)]. In the well-studied case of *alcR*, a "double-lock mechanism" occurs, where both the regulatory gene and the structural genes are directly controlled by CreA (Felenbok et al. 2001; Kulmburg et al. 1993). Furthermore, AlcR and CreA binding sites are overlapping in some co-regulated genes and CreA also acts by competition with AlcR for DNA-binding (Mathieu and Felenbok 1994; Mathieu et al. 2000). Competition for the same DNA-binding sites in the *amdS* promoter may occur between CreA and the Cys2His2 zinc finger pathway-specific activators AmdA and AmdX (Andrianopoulos et al. 1997; Murphy et al. 1997). Competition has also been proposed between CreA and a

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positive-acting factor in the *prnB-D* intergenic region (Gonzalez et al. 1997).

5 THE PROMOTER, GENE CLUSTERING, AND CHROMATIN REMODELING

The promoter of a metabolic structural gene provides a platform for modulating gene expression. Promoters comprise a regulatory region, which contains DNA-binding sites for the global and pathway-specific regulatory factors, and core elements for interaction of the general transcription machinery. Mutational and molecular studies have defined the arrangement of regulatory DNA-binding sites in the *amdS*, *prnB-C*, and *niiA-niaD* promoters (Davis et al. 1993; Punt et al. 1995; Scazzocchio 1992). Transcription factors may exert their effects directly on the general transcriptional machinery or indirectly by altering chromatin structure. Many metabolic pathway genes are clustered in fungi (Cary et al. 2001). Gene clustering allows coordination of changes in chromatin structure for co-expressed genes.

Regulation of the expression of genes encoding the nitrate utilizing enzymes in *N. crassa* and *A. nidulans* are well-documented examples (Caddick et al. 1994; Marzluf 1997). Nitrate is taken up by permeases, and reduced to nitrite by nitrate reductase. Nitrite is further reduced to ammonium by nitrite reductase. Genes encoding these proteins are organized differently in several fungi (Cary et al. 2001). For example, the nitrate permease (*crnA* = *nrtA*), nitrate reductase (*niaD*), and nitrite reductase (*niiA*) genes in *A. nidulans* are clustered (Johnstone et al. 1990) whereas the homologs of *niaD* and *niiA* in *N. crassa*, *nit-3*, and *nit-6*, respectively, are located on separate chromosomes.

Two NIT4 DNA-binding sites of significantly different affinities have been identified in the 1.3-kb promoter region of the *nit-3* gene (Fu et al. 1995). The stronger binding site contains the palindromic sequence, TCCGCGGA, whereas the weaker site has a related sequence (Fu et al. 1995). NIT2 binds strongly to regions in the promoter of *nit-3* containing at least two GTAT elements, which are 30 bp apart from each other (Chiang and Marzluf 1994). NIT2 DNA-binding site II, which contains only a single GATA element, also plays a major role in *nit-3* gene expression (Tao and Marzluf 1998). Thus, all the NIT4 DNA-binding sites and GATA elements in the *nit-3* promoter serve a regulatory function for nitrate utilization. Interestingly, a direct interaction between NIT2 and NIT4 is essential for optimal *nit-3* expression (Feng and Marzluf 1998), but the NIT2 and NIT4 binding sites can be separated without affecting either the expression level or the precise regulation of the *nit-3* gene (Tao and Marzluf 1998).

In the *A. nidulans niaD-niiA* intergenic promoter region, all four NirA DNA-binding sites, 5′-CTCCGHGG-3′ (H = A, C or T), have a regulatory function. It appears that site 1 is necessary for the inducibility of *niiA* alone, while sites 2, 3, and 4 act bidirectionally (Punt et al. 1995). The binding of NirA to the promoter is dependent on nitrate induction (Narendja et al. 2002). Of the ten AreA DNA-binding sites in the *niaD-niiA* intergenic region, the central clustered sites 5–8, which neighbor NirA DNA-binding site 2, are responsible for more than 80% of the transcriptional activity of both genes (Muro-Pastor et al. 1999). It appears that AreA is essential for chromatin remodeling (nucleosome repositioning) of the *niaD-niiA* intergenic region, and this process is independent from NirA-mediated transcription activation (Muro-Pastor et al. 1999). However, AreA is required for DNA-binding by NirA (Narendja et al. 2002). Thus, one function of AreA is to open the chromatin structure in the promoter region to allow access for NirA and general transcription factors to initiate gene expression. The chromatin arrangement also appears important at other promoters. In the *A. nidulans amdS* and *fmdS* promoters, the AnCF CCAAT-binding complex and CCAAT sequence [see Brakhage et al. (1999) for a review] are necessary for formation of nucleosome-free DNase I-hypersensitive sites and determine basal transcription levels (Fraser et al. 2001; Narendja et al. 1999).

6 CONCLUSIONS

Great progress has been made in the past decade in our understanding of the combined action of global and pathway-specific regulatory circuits, which mediate the preferential utilization of nitrogen and carbon sources, especially in the model fungi *A. nidulans* and *N. crassa*. A major challenge of future research is to understand how binding of a global regulator to a promoter modulates gene expression and how pathway-specific regulators recruit co-activators to a promoter to initiate gene transcription. Other challenges include elucidation of the sensors of nutritional limitation or sufficiency and the signal transduction systems and inducer molecules that relay these signals to the key effectors of the transcriptional response. The powerful combination of genetics, biochemistry, molecular and cell biology approaches and the recent characterization of novel regulatory genes and mechanisms have provided the framework to overcome the missing links in nitrogen and carbon metabolic gene regulation.

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4

Fungal Nuclei and Chromosomes

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1 INTRODUCTION

Since George Beadle and Edward Tatum (1941) experimented on genetic controls of biochemical processes using the fungus *Neurospora crassa* as a model system, fungi have taken the center stage for research in genetics, molecular and cellular biology, and more recently molecular cytogenetics. The unicellular yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have gained wide acceptance by many to be the organism of choice besides *Neurospora* and *Aspergillus*. In retrospect, progress appears to have followed the innovation of new technologies. For example, the creation of temperature-sensitive mutations has made it possible to analyze genes and their functions, gene manipulations can create truncated genes whereby specific domain and its function may be analyzed, and the yeast two-hybrid system provides a powerful tool to study protein–protein interactions. More recently, fusion proteins, created with green fluorescent protein (GFP), yellow fluorescent protein (YFP), and the like, have allowed live subcellular localization of specific proteins in question under confocal and/or fluorescence microscopy. For more accurate subcellular localization under electron microscopy, Myc- or Protein-A conjugates, or Glutathion-S-transferase (GST) conjugates have been used in conjunction with indirect immunogold localization. In addition, fluorescence *in situ* hybridization (FISH) has allowed localization of specific DNA or RNA species, such as mRNA transport or localization of telomere clusters (see references in this chapter). In the last six or so years, there has been an enormous information explosion in the area of fungal nuclei and chromosomes, especially the nucleolus, the spindle pole body (SPB), the nuclear pore complex (NPC), the centromere and the telomere. It is most gratifying to have the privilege to provide a brief review on these findings, but it is also with a humbling sense of inadequacy that many works may not be included for the lack of time and space. For those authors that I missed, my sincere apologies.

2 A REFLECTION OF TIMES—A PERSONAL ANECDOTE

When I started my graduate studies, my professor H.J. Brodie asked me what I would like to do for research. At that time I was fascinated by studies of meiosis of *N. crassa* (McClintock 1945; Singleton 1953) and *Sodaria fimicola* (Carr and Olive 1958), and there were no clear pictures of chromosomes of the Basidiomycetes. What attracted my attention the most was a review by Lindsey Olive (1953) that, without exception, every species of the Basidiomycetes ever studied had four chromosomes. Since Brodie's main interest involved the genus *Cyathus*, I offered to study the chromosomes of the Bird's Nest fungus. He told me to forget the idea because he said, "I had two internationally renowned cytologists look at it once and they could not see anything" instead, he wanted me to study the effect of light on fruiting and I did (Lu 1965). The rebellion inside me, however, wanted to try anyway. I first developed a staining technique using *Neurospora* (Lu 1962) as a test case. As I got *Cyathus stercoreus* to fruit, I prepared a slide, and was surprised to see some chromosome figures but the biggest prize of all was that I found, on a second look at the slide the following day, one nucleus showing 12 clearly separated chromosomes of *C. stercoreus* (Figure 1B) and we wrote a letter to Nature (Lu and Brodie 1962). When I cut out

*I wish to dedicate this chapter to Ms Iris Hseung who showed me, during my freshman year at National Taiwan University, that Biology is a beautiful area to explore.

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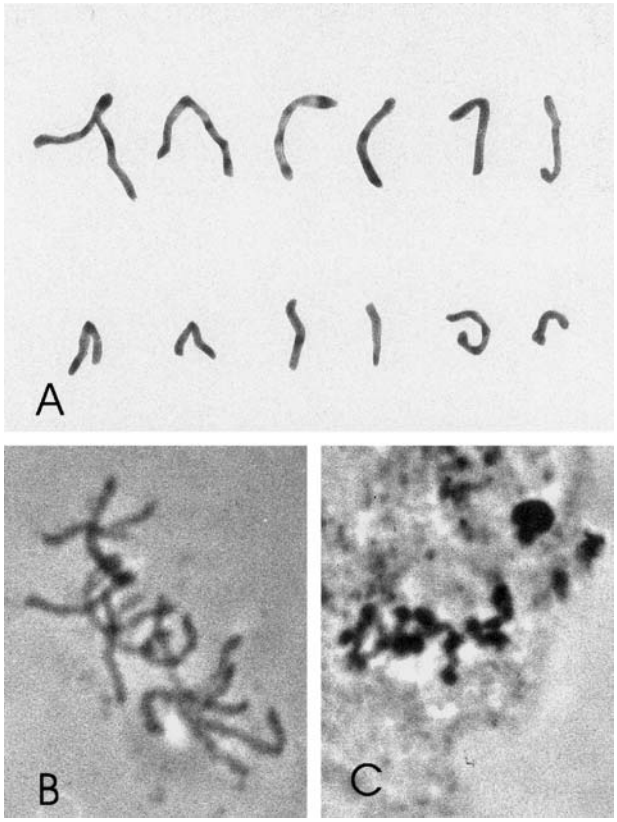

Figure 1 Chromosomes of *C. stercoreus*: A, 12 cut out chromosomes from Figure 1B and C, diakinesis showing quadrivalents for $1+2$, $3+4$, $5+6$, $7+8$, and $9+10$. Reproduced from Lu (1964).

all 12 chromosomes and aligned them according to sizes (Figure 1A), I was intrigued by the similarity between chromosomes 1 and 2, 3 and 4, 5 and 6, etc. I decided to look for an answer. I discovered that chromosomes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10 formed quadrivalents (Figure 1C) and I realized that I was dealing with a tetraploid Basidiomycete (Lu 1964).

In the early sixties, it was commonly thought that filamentous fungi have no mitosis and that chromatin simply separated into two tear-drop-like lumps (Robinow and Bakerspigel 1965). However, I found prophase chromosomes with associated nucleolus and the SPB in the mycelium (Figure 3B). I also found metaphaselike figures with two SPBs as well as anaphase–telophase figures. I presented my findings in a conference. After my presentation, a professor of cytology glared down at me and said, "Don't make any more chromosomes of fungi!" Times have changed and advances made and research in fungi is leading the way on many fronts in genetics, molecular and cellular biology, and recently in molecular cytogenetics. This is especially worth noting in analyses of different components of the nucleus and the chromosome. Fungal chromosomes, mitosis and meiosis have been reviewed (Lu 1996) and will not be repeated here. In this chapter, emphases will be given to some of the recent advances in nuclear organelles.

3 THE NUCLEOLUS

The nucleolus is the largest and most conspicuous body in the nucleus. It can be easily visualized under a light microscope by the help of a simple basic stain, such as acetocarmine or propiono-hematoxylin (Lu 1967a; Raju 1978). All fungi appear to have only one nucleolar organizer (NOR) located in a specific chromosome, which is known as the nucleolar chromosome. For example, it is chromosome 2 for *N. crassa*, (McClintock 1945), chromosome 10 for *Coprinus cinereus* (Pukkila and Lu 1985) and chromosome 12 for the yeast *S. cerevisiae*. A translocation that occurs at or near the NOR in *N. crassa*, such as T(IL-VL)OY321 and T(V-IV)AR33, could organize two nucleoli (Perkins et al. 1980; 1984). In the fission yeast *S. pombe*, where mitosis is intranuclear, the nucleolus persists throughout mitosis and eventually divided into the daughter cells (McCully and Robinow 1971). Under the squashed preparation, the NOR is often seen as a highly condensed body associated with the nucleolus. By FISH technology, the rDNA of *S. cerevisiae* exhibits changes according to the cell cycle, i.e., decondensed and dispersed in G1 and highly condensed as a distinct looped or linelike structure in M-phase (Lavoie et al. 2002).

3.1 Nucleolar Structure

With the help of electron microscopy, the nucleolus is seen to have substructures—the fibrillar component, the granular component (GC), the DNA containing component, and sometimes nucleolar vacuole. Actually, the subcompartmentalization was described in animal cells much earlier by Estable and associates in the early 30s and 50s using acid fixation and silver impregnation techniques. These authors described the "stranded network in a generally amorphous background" and they named it nucleolonema and pars amorphus. Their finding was not well received initially and the skeptics criticized it as an artifact because of the "nasty" treatments that were used. At the International Symposium on Nucleolus held in Montevideo, Uruguay, Estable (1966) demonstrated the same network in stained preparations and in live specimens under phase contrast microscopy. In fact, the clearest demonstration of nucleolonema was that of corn pollen mother cells provided by Swift and Stevens (1966). In light of recent studies with *Locusta* oocytes by conventional electron microscopy as compared with the Miller spreads (Scheer et al. 1997), the nucleolonema in the intact nucleus really represent the "Christmas trees" showing rRNA transcription in progress so elegantly demonstrated by Miller and Beatty (1969).

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3.1.1 Nucleolar Subcompartmentalization

The presence of nucleolonema has recently been confirmed by using cryofixation and cryosubstitution techniques in yeast, *S. cerevisiae* and *S. pombe* (Léger-Silvestre et al. 1997; 1999), an example of which is shown in Figure 2A. These authors demonstrated three major compartments of the nucleolus: the dense fibrillar component (DFC), the fibrillar center (FC) and GC. Moreover, they demonstrated that the FC contains rDNA by *in situ* hybridization (after RNase treatment) using the cloned 35S rDNA as a probe that was labeled with digoxigenin-11-UTP and the probe was detected by antidigoxigenin antibody-gold conjugate. They found that the gold particles are concentrated around the FC (Figure 2B). In the same token, they demonstrated that the nascent rRNA transcripts are mainly located in the DFC and never in the FC regions. This is proven by colocalization of three key rRNA transcription and processing proteins in the DFC regions: RNA polymerase I, which transcribes the pre-rRNAs, Gar1p, which is required for pseudouridylation of yeast rRNAs, and Nop1p, which is a component of the box C/D and plays a role in pre-rRNA methylation. The granular particles are preribosomes that eventually are transported to the cytoplasm through the nuclear pores. The same results were obtained in mouse cells using the 5′ external transcribed spacer (5′ETS) as a probe for *in situ* hybridization, which showed that nascent rRNA transcripts are restricted to the DFC regions (Puvion-Dutilleul et al. 1997).

It is interesting that yeast mutants, deleted of the rDNA repeats (but carrying many copies of a plasmid that includes a single rDNA sequence), showed no FC and no nucleolonema (Trumtel et al. 2000). Obviously, the rDNA sequences carried by the plasmids are not silenced and do not organize the FC zones or the nucleolonema, probably because they are not in tandem repeats. It is now clear that the chromosomal localization of the NOR containing tandem repeat of 100–200 copies of rDNA sequence is not essential for the transcription and processing of rRNAs or for recruiting

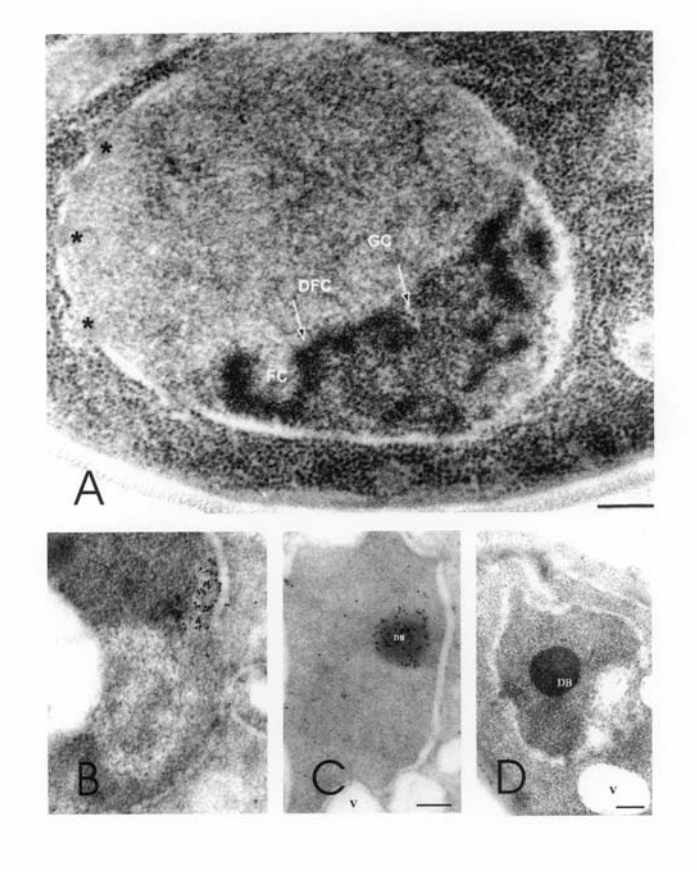

Figure 2 Electron micrographs of yeast nucleus showing nucleolar subcompartmentalization: A–B—from *S. cerevisiae* showing FC, GC, and DFC (A) and rDNA localization only in the FC (B); C–D—the nucleolus of *S. pombe* showing NDB from gar-1 mutant cells, the truncated Gar2p is accumulated in the DB (C). The bar = 1 μm Reproduced from Chromosoma 1997, 105:542–552 and Chromosoma 1999, 108:103–113, by copyright permission of Springer-Verlag.

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ribosomal proteins to the nuclear site (Nierras et al. 1997; Trumtel et al. 2000). However, the transcription by RNA pol I appears to be one of the main requirements for nucleologenesis (Trumtel et al. 2000).

3.1.2 The Nucleolar Dense Body (NDB)

The NDB or simply nucleolar body (NB) or dense body (DB) is another prominent fibrillar compartment that has not received due recognition. It was initially noted only in meiotic nuclei, in the wild-type *C. cinereus* (Lu 1984; 1996) and in yeast mutants (Horesh et al. 1979). It was never observed in mitotic nuclei. However, in a fission yeast mutant with a truncated *GAR2*$^{-}$ nucleolar protein gene, the nucleolus is reorganized to display a prominent NDB in mitotic cells and the defective gar2 protein is localized only in this NDB (Figure 2C and D); no rRNA is located in this compartment (Léger-Silvestre et al. 1997). In a separate case, budding yeast cells overexpressing the box C/D snoRNAs from multicopy vector also exhibit the additional NB structure in the nucleolus and the snoRNAs are seen to localize in this body as shown by electron microscopy and by *in situ* hybridization (Verheggen et al. 2001). These authors also showed similarities between NB of yeast and the Cajal or coiled body (CB), which exist in normal vertebrates (Gall 2000). These observations raise the question of whether or not the organization of this NDB compartment is related to some specific function(s), be it in meiotic or in mitotic nucleolus. For example, the central components of the synaptonemal complex were observed to assemble in the NDB in meiotic nucleolus (Haskins et al. 1971; Lu 1984).

3.2 Nucleolar Functions

The nucleolus is known to be the site of rRNA transcription and processing, i.e., the 35S rRNA was first transcribed from rDNA and then processed into 18S, 25S, and 5.8S rRNAs. These are combined with 5S rRNA and ribosomal proteins to form preribosomal particles for export to the cytoplasm (reviewed in Shaw and Jordan 1995). A nucleolar protein Rlp7p of the budding yeast is required for processing 27S rRNA into 5.8S and 25S rRNAs for the large subunit; defect in Rlp7p results in accumulation of 27S RNA in the nucleolus (Dunbar et al. 2000). Another essential and highly conserved protein, Zpr1p, has been identified in yeast. It is a cytoplasmic Zinc finger protein that accumulates in the nucleolus of proliferating cells and it may be involved in the regulation of 35S rRNA transcription (Galcheva-Gargova et al. 1998). It is possible that the nucleolus has other important functions that are not commonly known.

3.2.1 The Nucleolus May Be a Depot for MRNA Exports from Nucleus to Cytoplasm

The earliest hint that the nucleolus has functions other than ribosome biogenesis came from pioneering studies in the RNA transcription and transport from the nucleus to the cytoplasm in embryonic chick erythrocytes as well as in HeLa cells (Sidebottom and Harris 1969). These authors found that the RNA transport occurs only after the embryonic chick erythrocytes develop the nucleolus, suggesting that the nucleolus may play a role in the RNA transport. To confirm this hypothesis, they irradiated the nucleolus of chick erythrocytes as well as that of the HeLa cells with a microbeam of UV light and found that the RNA transport is impaired while the control cells (unirradiated, extronucleolar area of the nucleus irradiated, or even one of the two nucleoli irradiated) show normal or near normal RNA transport. The effect of the nucleolus on RNA transport is not limited to rRNAs, mRNA transport is also affected by UV irradiation of the nucleolus (Deak et al. 1972). These authors concluded that "some function located at or near the nucleolus is essential for the full expression of structural genes." After nearly three decades of silence, molecular biology finally brought evidence to support their claims (Schneiter et al. 1995).

To study the role of the nucleolus in mRNA export from the nucleus to the cytoplasm, genetics and molecular cytology are the most powerful tools, especially valuable are the temperature sensitive mutations, and the yeasts are the organisms of choice. A large number of temperature sensitive mutants have been isolated that are defective in mRNA transport at the nonpermissive temperature. The genes are named *MTR* for Messenger RNA TRansport genes (Kadowaki et al. 1994). In addition to its known function for rRNA transcription, processing and export, the studies that follow have demonstrated that the nucleolus is decisively involved in the mRNA export.

The indication that the nucleolus plays a role in the mRNA export came from temperature sensitive (ts) *MTR* mutants that are defective in mRNA transport at 37°C. In *S. cerevisiae*, *MTR*3 codes for a 28-KDa protein that appears to be a part of the mRNA transport machinery and *mtr*3-1 is a ts mutant. At 37°C, *mtr*3-1 failed to transport mRNAs to the cytoplasm resulting in an accumulation of polyA^{+} RNAs in the nucleus. By *in situ* hybridization using oligo-$(dT)_{50}$ as a probe, accumulation of polyA^{+} RNAs is restricted only to the nucleolus (Kadowaki et al. 1995). Since the wild-type cells show poor presence of polyA^{+} RNA in the nucleoplasm and in the nucleolus, the transport pathway is uncertain. However, a clearer picture is shown in the fission yeast, *S. pombe*, by electron microscopic *in situ* hybridization with an oligo $(dT)_{50}$ probe. Poly$(A)^{+}$ RNAs are uniformly distributed in the chromatin-rich region, in the nucleolar region, as well as in the cytoplasm in control cells at 30°C, whereas poly$(A)^{+}$ RNAs are accumulated in the fragmented condensed nucleolar regions in heat-shocked cells, suggesting that exports of mRNA normally pass through the nucleolus (Tani et al. 1996). Under severe heat-shock conditions, that impair RNA transport, accumulation will occur at the location where the last stop of the transport pathway is, i.e., the nucleolus. In *S. pombe*, *pim*1, which has a defect in mRNA transport at 37°C, also accumulates poly$(A)^{+}$ RNA in the nucleolar region (Tani et al. 1996).

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Further evidence came from the cellular localization of Mtr3p. A gene encoding Mtr3p with the HA epitope at its carboxy terminus was constructed in YTK306 strain (with tagged Mtr3p). By subcellular fractionation and by immunofluorescence microscopy, HA-epitope-tagged Mtr3p cofractionates with the nucleolar-protein Nop1p, which is required for rRNA processing, and colocalizes with Rpa190p, which is the largest subunit of RNA polymerase I confirming that Mtr3p is localized in the nucleolus (Kadowaki et al. 1995). Since Mtr3p is involved in the mRNA export and it is located in the nucleolus, it seems reasonable to conclude that the nucleolus is a depot for mRNA exports.

3.2.2 The Nucleolus is the Site of Signal Recognition Particle (SRP) Assembly and Export

The secretory and membrane proteins are targeted to the endoplasmic reticulum (ER) membrane vesicles by the SRP because the nacent polypeptides of these proteins all carry a signal sequence. All SRP proteins, except one, are concentrated in the nucleolus as shown by fluorescence microscopy (Grosshans et al. 2001). Other RNA factors for RNA processing such as snRNA, snoRNA, and box C/D proteins are also known to use CB/NB and/or nucleolus as transitory station before reaching their target sites (Verheggen et al. 2001).

3.2.3 The Nucleolus May Hold Keys to Cell Cycle and Check Point Controls

In eukaryotes, the maintenance of genome integrity is achieved through the operation of cell-cycle checkpoints that ensure the completion of one cellular event before the initiation of another, and the checkpoint controls operate in mitotic as well as in meiotic cells (Hartwell and Weinert 1989; Murakami and Nurse 2000). In meiotic prophase, the main event is the synapsis of homologous chromosomes and genetic recombination between nonsister chromatids. Failure to complete one or both events, such as in *dmc*1, *zip*1, or *hop*2 mutant of the yeast, *S. cerevisiae*, will lead to slowing down of meiotic progression and eventual arrest at pachytene; this control mechanism has been named pachytene checkpoint (Roeder and Bailis 2000). Genes that participate in the checkpoint controls can be identified because their mutations cause a bypass of pachytene arrest. A number of checkpoint proteins and their genes have been identified (Roeder and Bailis 2000). Here, only three proteins will be discussed, as they are closely associated with the nucleolus, and they are Dot1p (also known as Pch1p), Pch2p, and Sir2p; all three are silencing factors and all three are nucleolar proteins. In mitotic cells, using the help of the green protein, Dot1p-GFP, the signal is located in the nuclear region not stained by DAPI, i.e., the nucleolus. This nucleolus accumulation is predominant in 70% of the cell population. The nucleolar localization is confirmed by colocalization with the nucleolar protein Nsr1p (San-Segundo and Roeder 2000). In meiotic cells, Dot1p is uniformly distributed in all chromatin regions. In contrast, the meiosis-specific protein Pch2p and chromatin-silencing factor Sir2p are localized predominantly in the nucleolus (San-Segundo and Roeder 2000). It is most interesting that the nucleolar localization of Pch2p is essential for the checkpoint to be operative (e.g., in *zip*1 cells), when Pch2p is delocalized from the nucleolus (e.g., in *sir*2 mutant cells), the checkpoint is inactive (Roeder and Bailis 2000). It appears that Dot1p is required for the nucleolar localization of Sir2p, which in turn is required for the nucleolar localization of Pch2p. This is shown in the *zip*1 *dot*1 double mutant that bypassed the pachytene arrest and in which Pch2 proteins are mislocalized throughout the chromatin regions (San-Segundo and Roeder 2000).

The nucleolus may also have a role in the mitotic cell-cycle controls. There are three transitions in mitotic cell cycles: G1 to S-phase, metaphase to telophase, and the exit from mitosis. The key players are genes for the cyclin-dependent kinases (*CDK*s) and their associated stage specific cyclins, namely, G1 cyclins (*CLN*1, *CLN*2, and *CLN*3), S phase cyclins (*CLB*5 and *CLB*6), and mitosis cyclins (*CLB*1 through *CLB*4), and the key kinase is $p34^{cdc2/cdc28}$, which is also known as maturation promoting factor (MPF): *Cdc*2p for the fission yeast and Cdc28p for the budding yeast (King et al. 1996; Visintin et al. 1997). Activation of this kinase system promotes the entry to mitosis and its inactivation leads to exit from mitosis (Visintin et al. 1999). The point of interest where the nucleolus plays a part is the exit from mitosis which, in the budding yeast, is controlled by two redundant mechanisms to inactivate Cdc28—by proteolysis of the mitotic cyclin Clb2p, and by accumulation of Cdc28p/Clbp inhibitor Sic1p that binds and inhibits the kinase activity (Visintin et al. 1997; 1999). Both processes are controlled by the anaphase-promoting complex (APC) and its substrate-specific activator Cdc20p and Cdh1p/Hct1p. Cdc20p controls the metaphase–anaphase transition and Cdh1p/Hct1p controls the exit from mitosis by degrading mitotic cyclin B (Visintin et al. 1997). In addition to APC, another set of genes is essential and these genes are collectively referred to as the mitotic exit network (MEN). They include *TEM*1, *LTE*1, *CDC*15, *DBF*2/*DBF*20, *CDC*5, *MOB*1, and *CDC*14 (Morgan 1999). Inactivation of any one of these genes leads to cell-cycle arrest at late anaphase–telophase (Visintin et al. 1998). Of the MEN, Cdc14p is a dual specificity protein phosphatase and it may be the key player because over expression of Cdc14p causes degradation of Clb2p and accumulation of Sic1p (Visintin et al. 1998). Cdc14p is sequestered to the nucleolus by a nucleolar protein, Cfi1p (for Cdc14 inhibitor; Visintin et al. 1999), which is also known as Net1p (for nucleolar silencing establishing factor and telophase regulator; Shou et al. 1999). Net1p(Cfi1p) is an inhibitor and a negative regulator of Cdc14p (Visintin et al. 1999; Shou et al. 1999). Net1p(Cfi1p) forms a complex with both Cdc14p and Sir2p and the Cdc14p-Net1p-Sir2p complex is referred to as "RENT" for regulator of nucleolar silencing and telophase (Shou et al. 1999). Net1p is responsible for anchoring Cdc14p to the nucleolus, where its phosphatase activity is suppressed during

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G1, S, and M phase of the cell cycle (Shou et al. 1999; Traverso et al. 2001). To facilitate exit from mitosis, Cdc14p is released from the nucleolus and is activated during late anaphase/telophase, when the spindle is elongated, but Net1p remains in the nucleolus. Here the nucleolus functions as a sequestation center, by the help of Net1p, to prevent premature exit from mitosis Bachant and Elledge 1999; Shou et al. 1999; Visintin et al. 1999).

What controls the release of Cdc14p from RENT? When the budding yeast was synchronized and the localization of Cdc14p was monitored at 10 min intervals, a clearer picture emerged. Cdc14p is transiently released from the nucleolus, not only in the wild type cells, but also in the majority of the MEN mutants, except in *cdc*5-1 cells. By using this assay, a number of mutants were identified that are defective in the MEN-independent transient release of Cdc14p and these are *esp*1, *slk*19, and *spo*12 as well as *cdc*5-1. Thus, the transient release of Cdc14p from the nucleolus at early anaphase requires *ESP*1, *CDC*5, *SLK*19, and *SPO*12 that collectively are named the fourteen early anaphase release (Cdc FEAR) network of which *ESP*1 may be the major player (Stegmeier et al. 2002). The FEAR network is not essential because mutation in any one of its components leads only to a delay in exit from mitosis. However, it is absolutely essential for release of Cdc14p and exit from mitosis in cells lacking *LTE*1, for *esp*1-1 *lte*1Δ, *lte*1Δ *slk*19Δ, and *spo*12Δ *lte*1Δ double mutants are inviable (Stegmeier et al. 2002). Taken all together, the sequence of events may be summarized as follow: (a) Cdc20p of APC controls the metaphase–anaphase transition, (b) the FEAR network initiates the transient release of Cdc14p from the nucleolus during early anaphase and activates its phosphatase activity that dephosphorylates Cdc15p and activates the MEN network, (c) MEN maintains Cdc14p in the permanent released state at late anaphase–telophase, (d) the active Cdc14p phosphatase dephosphorylates Cdh1p/Hct1p, Swi5p, and Sic1p which in turn inactivate mitotic cyclin, Clb2p, and protein kinase, Cdk1p, to allow exit from mitosis, and (e) after mitosis, Cdc14p is returned to the nucleolus.

3.2.4 Nucleolar Silencing and Its Control of Aging

The main DNA component of the nucleolus is the nucleolus organizer, which contains 100–200 tandem repeats of rDNA whose transcription by RNA polymerase I is essential for the structural integrity of this organelle (Trumtel et al. 2000). In general, there is no recombination activity in these ribosomal repeats as exemplified in *C. cinereus* (Cassidy et al. 1984). The understanding of this unusual phenomenon has come to light in recent years through genetics and molecular cytological research especially in *S. cerevisiae*. Yeast cells divide asymmetrically (the mother is larger than the daughter) and there is a finite number of daughters each mother can produce before senescence sets in and this is controlled by silencing factors. A number of genes have been identified that control silencing in the telomere, in mating type loci (HML and HMR), and in the nucleolus. They are the silence information regulators (SIR) 2–4. Furthermore, silencing in the nucleolus appears to be related to aging (reviewed in Guarente 2000; Johnson et al. 1998; Shore 2000). By careful experimentation, Kaeberlein et al. (1999) provided evidence that although all *SIR* genes are involved in silencing, *SIR*2 appears to play a central role in yeast aging and it does so by preventing recombination in rDNA repeats. Recombination is the main cause of aging because it creates extrachromosomal rDNA circles (ERC), which are preferentially retained by the mother, as shown by elevated rate of ERC formation in *sir*2 mutants, which have a reduced life span by 50% (Kaeberlein et al. 1999). Moreover, the Sir2p appears to be the limiting factor, because a *SIR*2/*sir*2 heterozygous diploid has a shorter life span than the *SIR*2/*SIR*2 homozygote, and an integration of a second copy of *SIR*2 into the wild-type strain gave rise to an ~30% extension of life span (Kaeberlein et al. 1999).

Sir2p is immuno-localized to the nucleolus of yeast cells using affinity-purified antibodies against Sir2p. A strong Sir2p signal in the nucleolar domain is confirmed by its unambiguous colocalization with the nucleolus specific protein Nop1p (Gotta et al. 1997). A weaker punctate pattern of Sir2p signals is also found at or near the nuclear periphery. These signal foci are confirmed to be on the telomeres by virtue of their colocalization with the telomere binding protein, Rap1p (also known as repression activator protein). Thus, Sir2p is located mainly in the nucleolus. In a somewhat lesser way, it is also located in the telomeres. The association of Sir2p with the telomeres is lost when Sir3p, Sir4p, or Sir2p are mutated (Gotta et al. 1997). Sir3/4p are not DNA binding proteins, they are recruited to the telomeres by Rap1p (Moretti and Shore 2001). In the old cells, the Sir3p proteins are redirected to the nucleolus and the proper relocalization to the nucleolus requires Sir2p as well as yet another protein Uth4p (Gotta et al. 1997; Sinclair et al. 1997). Kaeberlein et al. (1999) suggested that the telomeres may be holding a regulatory balancing act and that the relocalization of the Sir proteins from the telomeres to the nucleolus in the old cells is to counter aging (Gotta et al. 1997; Johnson et al. 1998; Kaeberlein et al. 1999). Indeed, the Sir3p redirection to the nucleolus was shown to delay aging process (Sinclair et al. 1997).

4 THE SPINDLE POLE BODY

Before the electron microscope (EM), the SPB was variously called centrioles, centrosomes, and, after EM, kinetochore equivalent (KCE) or microtubules organizing center (MTOC). At the first international mycological congress, the term SPB was proposed. It is equivalent to the centrosomes of the animal cells in the functional sense. In fungi, it is an organelle closely associated with the nucleus (Figure 3A and B) but located outside of the nuclear envelope (NE) (Girbardt 1971; Raju and Lu 1973). During mitosis and meiosis, the SPB divides at prophase and moves to the opposite poles at metaphase to help organize the spindle. In Basidiomycetes, the SPB is globular in shape before division,

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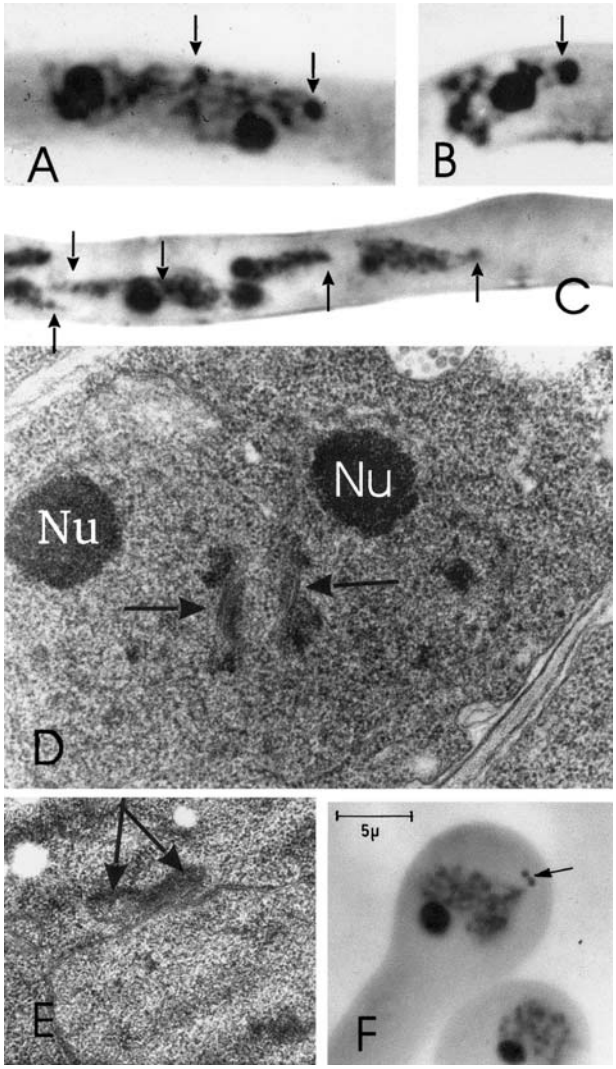

Figure 3 A–B—prophase nuclei in the mycelium of *N. crassa* each contains a nucleolus and an SPB (arrowed); C—*N. crassa* showing "horse tail" nuclei traveling to and from the hyphal tip with the SPB at the leading edge, traveling right to the tip (up arrows), and to the left on return flow (down arrows); D—electron micrograph (EM) of a basidium of *C. cinereus* during karyogamy showing two fusing haploid nuclei at the point of contact between two respective SPBs (arrowed); note the darkly stained chromatin (centromere clusters?) from each nucleus are situated symmetrically opposite each other; Nu, nucleolus; E—EM image of a diploid nucleus at diplotene showing divided SPBs; F—diplotene nucleus showing divided SPBs, reproduced from Raju and Lu (1973).

and is dumbbell-shaped during division and its morphology does not change in mitosis or in meiosis (Raju and Lu 1973). In the Ascomycetes, such as *Neurospora*, *Gelasinospora*, and *Podospora*, etc. the SPB changes from a small rod in meiosis I to a large plaque in division II and III in the ascus (Lu 1967a; Raju 1978; Raju 1980; Zickler 1971). In addition to spindle organization, the SPB also help delimit the spore formation at the end of division III in the ascus (Lu 1967b; Raju 1980).

4.1 The SPB is At the Leading Edge of Nuclear Migration

When *N. crassa* was grown on a thin layer of agar medium on a microscope slide and examined live under a phase contrast microscope, rapid protoplasmic streaming to and from the hyphal tips was observed. When this slide culture was fixed and processed, the SPB was seen located at the tip of what is recently described as a "horse tail" nucleus suggesting that the SPB is leading the way with the nucleolus trailing behind (Figure 3A–C). Recent studies using fluorescence staining demonstrated that Kms1p, a component of the SPB of *S. pombe*, is located at the leading edge of the two traveling haploid nuclei to be fused at karyogamy (Niwa et al. 2000; Chikashige et al. 1994). Moreover, nuclear migration in the budding yeast is controlled by the core SPB component Nud1p as nuclear migration is defective in *nud*1-2 mutant cells (Gruneberg et al. 2000). Thus, the SPB does lead the movement during nuclear migration.

Meiosis in the basidia is initiated by the fusion of two haploid nuclei. It is possible that the SPBs lead the movement to consummate karyogamy in *C. cinereus*; Figure 3D depicts the two SPBs from two haploid nuclei positioned face to face just before nuclear fusion. The condensed chromatin symmetrically placed near the SPBs may be the centromere clusters (Figure 3D). The diploid nucleus should have two SPBs and this is observed in *C. cinereus* at diplotene by electron and light microscopy (Figure 3E and F; Raju and Lu 1973).

4.2 The SPB is Always Associated with the Chromosomes

The SPB is not just a part of the mitotic or meiotic spindle mechanism, it is intimately associated with the chromosomes even during interphase and prophase. Using a whole mount electron microscopy, a rare image was captured where chromosomes at prophase III of *N. crassa* were found to be intimately associated with the divided SPBs (Figure 4). In mitotic cells, the association is between the SPB and the centromere. However, during meiosis, nuclear reorganization occurs and the centromere association is replaced by the telomere clusters (Trelles-Sticken et al. 2000).

4.3 The SPB Plays a Role in MEN Regulation

Recent advances in molecular cytology, using GFP (or YFP) fluorescence microscopy, or using *in situ* immunofluorescence

microscopy, has made it possible to localize proteins in the SPB during mitosis. The results are most revealing. In *S. cerevisiae*, many of MEN components are localized to the SPB at anaphase/telophase and the SPB localization of Tem1p, Lte1p, Cdc15p and Dbf2p depends on the SPB core protein Nud1p. The recruitment appears to be orderly; Tem1p is recruited by Nud1p, Cdc15p by Tem1p, and Dbf2p by Cdc15p (Visintin and Amon 2001; Xu et al. 2000). Bub2p-Bfa1p/Byr4p complex localizes to SPBs constitutively by directly interacting with Nud1p (Xu et al. 2000). The SPB is a multilayered structure. It consists of an outer plaque, a central plaque and an inner plaque. The outer plaque is responsible for organizing cytoplasmic (astral) microtubules while the inner plaque is for nuclear microtubules. The central plaque is inserted in the NE [reviewed in Pereira and Schiebel (2001)]. It is now clear that the SPB has a role in MEN regulation and in the control of exit from mitosis.

It is interesting to note that, for its proper function, the SPB requires small GTPase Tem1p, its GDP/GTP exchange factor Lte1p and the GTPase activating protein (GAP) complex Bub2p-Bfa1p/Byr4p together with protein kinases Cdc15p and Dbf2p (Gruneberg et al. 2000; Visintin and Amon 2001). Taken together, the SPB controls of nuclear migration and of exit from mitosis may be energy requiring processes.

5 THE NUCLEAR PORE COMPLEX (NPC)

Like all eukaryotic cells, the fungal nucleus is enclosed by a double membrane system, called the NE, which differs from other double membrane systems in having many NPCs embedded in it. The NPC is a very specialized organelle, which is the gateway for all nucleocytoplasmic transports. NPCs of the budding yeast have been isolated and examined by transmission and by scanning electron microscopy (Rout and Blobel 1993). The NPC has an overall diameter of 97 nm, which is composed of eight interconnected spokes that span perpendicularly between the outer and inner nuclear membranes and arranged in an 8-fold symmetry forming the central ring. The center of this ring is the gate channel (or transporter). The radial length of each spoke is ~26 nm. Sitting over the gate channel is a plug of ~35 m in diameter that may have an aperture. The height of the NPC is ~30 nm, and the molecular mass is ~66 MDa. On the inner nuclear membrane is embedded a nucleoplasmic coaxial ring, attached to its periphery are eight long fibers of ~40 nm in length. These extend into the nucleoplasm to form a "nuclear basket." On the outer nuclear membrane, at the cytoplasmic face, is embedded another coaxial ring from which eight short fibers protrude into the cytoplasm [Rout and Blobel 1993; reviewed by Fabre and Hurt (1997)]. From purified NPCs, about 30 nuclear pore proteins have been identified, which are collectively named nucleoporins or nups, only three of these are pore membrane (Pom) proteins (Rout et al. 2000). A specific subset of these contains Phe-Gly repeats (FG nups) and appears to constitute most of the filamentous structures emanating from the NPC [cited by Rout et al. (2000)]. These nups have been found by

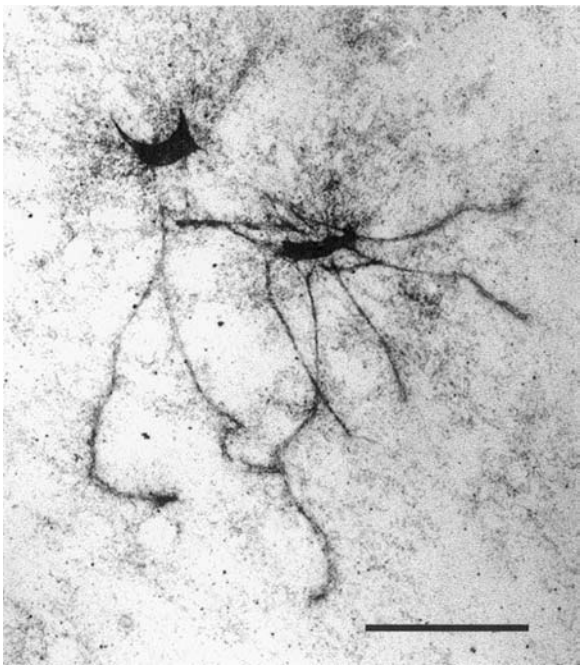

Figure 4 Whole mount electron microscopy of a prophase III nucleus from the ascus of *N. crassa*, showing divided SPBs and associated 7 chromosomes. The bar = 10 μm. (B.C. Lu, unpublished photo.)

immunolocalization to be symmetrically located between the nuclear and cytoplasmic faces; a few are biased toward nucleoplasm (Nup145N) or cytoplasm (Nup100p and Nup116p). Two FG nups (Nup1p and Nup60p) are exclusively nucleoplasmic that are significantly outward from the NPC mid-plane. These two proteins may constitute the nuclear basket. On the other hand, Nup159p, Nup42p, and Nup82p are exclusively cytoplasmic and they may be the cytoplasmic filaments. It is possible that these FG-nups provide docking sites for nucleocytoplasmic trafficking. The architecture of the NPC is shown in Figure 5 (Rout et al. 2000).

Nucleocytoplasmic exchange of macromolecules takes place through the NPCs and this transport is an energy dependent process. Many proteins are involved and these include Ran-GTP, some soluble factors for mRNA export (e.g., Mex67p, Mtr2p, Yrb2p), import/export receptors (e.g., Xpo1p), and proteins that shuttle their cargoes from the nucleus to the cytoplasm through the NE (e.g., the DEAD-box protein, Rip1p, etc). The DEAD-box protein Dbp5p(Rat8p) of the budding yeast is a highly conserved family of proteins and it is essential for mRNA export (Hodge et al. 1999). ProtA-Dbp5p is cytoplasmic and associated with the cytoplasmic fibrils (Strahm et al. 1999). In contrast, ProtA-Rip1p was detected in the nucleoplasm as well as on both sides of the NPC, in association with the nuclear basket and the cytoplasmic fibrils. It is interesting to note that its localization is not changed under the heat shock stress at 42°C (Strahm

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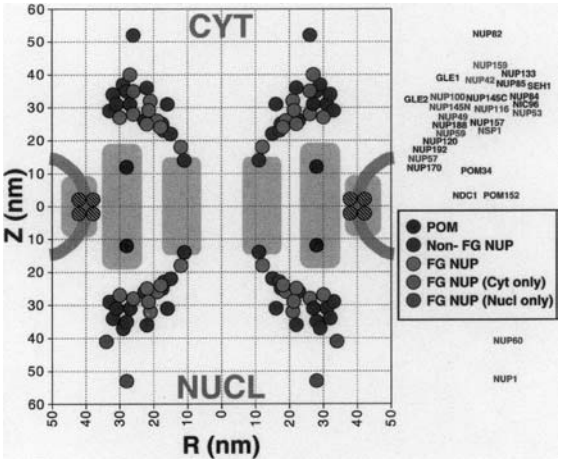

Figure 5 A model of nuclear pore complex by Rout et al. (2000), reader should consult the original in color for details. Reproduced from The Journal of Cell Biology, 2000, 148, 635–651, by copyright permission of The Rockefeller University Press.

et al. 1999). This is of particular significance because Rip1p is essential for heat-shock mRNA export when other export channels are shut down under stress (Saavedra et al. 1997; Tani et al. 1996). Taken all together, it is possible that on the cytoplasmic face of NPC, Nup159p may serve as the docking platform on which Dbp5p and other transport factors interact to mediate the late steps of mRNA export (Hodge et al. 1999; Snay-Hodge et al. 1998; Strahm et al. 1999; Rout et al. 2000). On the nucleoplasmic face of the NPC, Nup1p and Nup60p form the nuclear basket, which allows the trimeric Kap60p-Kap95p-Nup2p complex to dock to facilitate nuclear protein imports and exports; docking to Nup60p depends on the presence of Nup2p (Dilworth et al. 2001). Kap60p (Srp1p) is a member of the importin α, and Kap95p is a member of the exportin β, and they form heterodimers in association with Nup2p (Hood et al. 2000). Thus, whether it be the import or the export of Kap60p (Srp1p) and its cargoes, Nup2p is the key anchor at the NPC for bidirectional trafficking (Booth et al. 1999; Denning et al. 2001; Dilworth et al. 2001; Hood et al. 2000; Solsbacher et al. 2000).

6 CENTROMERES AND KINETOCHORES

The centromere is a very specific DNA sequence located in each chromosome to which a congress of centromere binding proteins are bound. This complex proteinaceous organelle is called the kinetochore, which is the nucleating site for the spindle microtubules (see review in Hyman and Sorger 1995). There appears to be only one microtubule per chromosome of the budding yeast (Peterson and Ris 1976), the same may be true for other fungi (Kubai 1975). The number of centromeres per haploid cell for any given species is highly controlled and addition of extra cloned copies is toxic to haploid yeast (Futcher and Carbon 1986; Runge et al. 1991).

6.1 Centromere Organization

The centromere sequences have been analyzed in the budding yeast (Bloom et al. 1983; Clarke and Carbon 1995). The centromere sequence of *S. cerevisiae* is the simplest among fungi. It contains a 220–250-bp sequence, which is divided into three functionally distinct sequences, namely CDEI, CDEII, and CDEIII. The CEDII is an A + T rich central region of 78–86 bp. A deletion of all or part of it leads to increased nondisjunction of chromosomes in mitosis and premature separation of sister chromatids in meiosis (Gaudet and Fitzgerald-Hayes 1989). CDEIII is the most critical one. A point mutation of the central C to T in the inverted repeat will abolish the centromere function and structure (Kenna et al. 1988). The centromere DNA sequence of *S. cerevisiae* is very small compared to that of the fission yeast *S. pombe* (Clarke and Baum 1990; Murakami et al. 1991), and *N. crassa* (Centola and Carbon 1994), the organization of which may be closer to those of the higher eukaryotes. It contains repeated sequences reminiscent of the pericentric heterochromatin of higher eukaryote [see Lu (1996) for diagrams].

6.2 The Kinetochore is a Complex Organelle

Although the centromere is simple, the kinetochore is complex with a large number of centromere binding proteins and associated proteins. It is made up of three distinctive compartments: the inner, the central, and the outer kinetochore [see review by Cheeseman et al. (2002)]. The centromere DNA of *S. cerevisiae* is organized into centromere specific nucleosomes that contain specialized histone H3-like proteins, Cse4p, which is a homolog of CENP-A. In the inner kinetochore, the most important one of the centromere binding proteins is the CBF3 complex (Ndc10p, Cep3p, Ctf13p, and Skp1p), which binds to CDEIII; without it, the kinetochore function is abolished. Other inner kinetochore proteins are Cbf1p/CENP-B, and Mif2p/CENP-C [see review in Cheeseman et al. (2002)]. There are many more proteins associated with the inner kinetochore proteins and these form the central kinetochore: the Ctf19 complex (Ctf19p, Mcm21p, and Okp1p), the Ctf3 complex (Ctf3p, Mcm16p, and Mcm22p) and the Ndc80 complex [Ndc80p, Spc24p, Spc25p, and Nuf2p; reviewed in Cheeseman et al. (2002)]. The most important one is the Ndc80 complex, without it the kinetochore function is defective.

6.3 Kinetochore Involvement in Spindle Organization

The function of the kinetochore is the organization of spindle microtubules during nuclear division and this is the function

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of the outer kinetochore where microtubules are attached by the help of microtubule-associated proteins (MAPs). The first kinetochore-associated MAP to be identified is Dam1p that is localized to the periphery of the SPB and clustered on the mitotic spindle as shown by immuno-electron microscopy suggesting that Dam1p is a component of the kinetochore. This is indeed the case as Dam1p is colocalized with the inner kinetochore component Ndc10p to the kinetochore on chromosome spreads by immunofluorescence microscopy (Jones et al. 2001). Dam1p plays a role in kinetochore attachment to the metaphase spindle and in anaphase spindle elongation (Jones et al. 2001). A large number of proteins are associated with Dam1p and this multiprotein complex is known as the Dam1p complex of the outer kinetochore (Dam1p, Duo1p, Dad1p, Spc19p, Spc34p, Dad2p, Ask1p, Dad3p, and Dad4p; Cheeseman et al. 2001; Janke et al. 2002; Cheeseman et al. 2002). It is also known as DASH (Dam1p, Duolp, Ask1p, Spc34p, Spc19p, and Hsk1p) complex (Li et al. 2002). This complex is localized to kinetochores in an Ndc10p- and Ndc80p-dependent manner [Enquist-Newman et al. 2001; Jones et al. 2001; see review in Cheeseman et al. (2002)]. Ask1p has been analyzed in detail and it was found to play an essential role in mitosis (Li et al. 2002). Unlike Dam1p, Ask1p is localized along the spindle as well as in kinetochores. It exhibits a single dotlike GFP signal in G1 cells and two dot signals in division stage near the spindle pole bodies when the spindle is elongated. The dotlike localization depends on the presence of the inner kinetochore CBF3 subunit Ndc10p, because in *ndc*10-1 mutants, the dotlike signals are mislocalized into a diffuse nuclear staining although the staining of Ask1p along the spindle is still present (Li et al. 2002). Further, Ask1p is unique among kinetochore proteins. It specifically associates with centromeres and its loading to kinetochores is dependent upon an intact spindle, because centromere binding of Ask1p is inhibited by nocodazole and this inhibition is reversible. It appears that the spindle microtubules are responsible for loading Ask1p to newly replicated kinetochores (Li et al. 2002). It is most interesting to note that *ask*1-3 mutants undergo massive nondisjunction without sister chromatid separation suggesting a defect in the spindle–kinetochore interaction that leads to failure in setting up a bipolar attachment (Li et al. 2002). Two more proteins were recently found to promote chromosome bi-orientation in mitosis (Tanaka et al. 2002). Ipl1p is the only Aurora kinase in *S. cerevisiae* and it forms a complex with Sli15p, a yeast ortholog of INCENP protein of animal cells. Both *ipl*1 and *sli*15 mutants shows high degree of chromosome missegregation with a strong bias of paired sister kinetochores connected to the old SPB in the bud of *S. cerevisiae* (Biggins et al. 1999; He et al. 2001; Tanaka et al. 2002). It is possible that Ipl1p-Sli15p complex is required to disrupt the spindle connection between the kinetochores and the old SPB to allow bi-orientation when new kinetochores are replicated (Tanaka et al. 2002). This hypothesis is consistent with the observations that Ipl1p-Sli15p complex is colocalized with CBF3 subunit Ndc10p for much of the cell cycle except for the metaphase–anaphase transition when Ipl1-GFP signals appear to move away from the kinetochores (Tanaka et al. 2002).

7 THE TELOMERES

Eukaryotic chromosomes are capped with a very specialized chromatin structure known as telomere. The telomere has two important functions: (a) to protect the integrity of the chromosome end, and (b) to ensure complete replication of the chromosomal DNA [reviewed in Blackburn and Szostak (1984), Zakian 1996, and Blackburn (2001)]. The telomere sequence was first identified in Tetrahymena as $(3'CCCCAA5')n/(5'TTGGGG3')n$ (Blackburn and Szostak 1984). Similar but not identical hexanucleotide repeats are found in other eukaryotes. In fungi, *S. pombe* has 5′-TTACAGG-3′, *N. crassa* has a hexanucleotide repeat of 5′ TTAGGG3′, a sequence it shares with human and other animals (Schechtman 1990). The yeast *S. cerevisiae* is different because its telomere sequence is degenerate; its sequence may be defined as $G_{2-3}(TG)_{1-6}$. Besides the hexanucleotide telomere sequence, there are subtelomeric (X and Y') sequences that are also important for the maintenance of telomere integrity (Zakian 1996).

7.1 Telomere Capping

Telomeres are capped and capping requires ~80–100 bp of genuine TG repeat sequence (Grossi et al. 2001). Uncapping of telomeres by incorporation of mutant DNA sequences leads to telomere deregulation and detrimental cellular consequences, like defects in cell division, in the yeast *Kluyveromyces lactis*. However, recapping can be achieved by addition of only a few wild-type telomeric repeats to their ends (Smith and Blackburn 1999). The proper telomeric TG repeat sequences are required for the binding of the repressor activator protein 1 (Rap1p), an essential telomere binding protein, to complete the cap formation where Rap1p binds specifically to the duplex telomeric repeats. The Rap1p binding is possible even when the repeat sequences are in the wrong orientation so long as the end is capped with the correct sequence orientation. It is interesting that the wrong orientation can be corrected by the *Rad*52-dependent recombination pathway (Grossi et al. 2001). It appears that Rap1p binding requires a minimum of two consecutive telomeric repeats having a 16 bp center-to-center spacing (Wahlin and Cohn 2002). Unlike human and fission yeast, where Rap1p is recruited by TRF proteins and Taz1p respectively, Rap1p of *S. cerevisiae* binds directly to the telomere repeats (Chikashige and Hiraoka 2001). The telomere sequence is further capped by other telomere binding proteins, namely, Ku proteins, Cdc13p, Stn1p, Ten1p, for the budding yeast [Grandin et al. 2001; Lin et al. 2001; Pennock et al. 2001; Wang et al. 2000; reviewed in Blackburn

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(2001)], and Pot1p and Ku proteins for the fission yeast (Baumann and Cech 2000; Baumann and Cech 2001).

7.2 Telomere Maintenance

Because of the biochemical nature of the DNA polymerases, the leading strand replication needs to be primed by RNA primer. Therefore, a few nucleotides will be lost in each round of replication. This is overcome by a specialized RNA-enzyme called telomerase (or telomere terminal transferase). This enzyme carries its own RNA template for the G-rich strand extension by a reverse transcription process. This enzyme is encoded by genes *EST*1-3 (for the polypeptide component) and *TLC*1 (for the RNA template). Mutations or deletion of these genes will cause ever-shorter-telomere phenotype and eventual senescence, known as replicative senescence. It was found that yeast cells lose ~200 nt (in *tlc*-Δ), and ~180 nt (in *yku*70-Δ) in 60–80 generations, or three successive restreaks of a colony (Förstemann et al. 2000). However, in the absence of telomerase, two parallel bypass pathways involving *SGS*1 and *RAD*50/51 genes could generate survivors (Johnson et al. 2001).

Cdc13p is a single-strand and sequence-specific telomere binding protein of *S. cerevisiae* (Lin et al. 2001; Pennock et al. 2001; Qi and Zakian 2000) while Pot1p is for *S. pombe* (Baumann and Cech 2001). Cdc13p is an essential protein as *cdc*13-null mutants are lethal (Pennock et al. 2001). Cdc13p interacts with Est1p of telomerase, with DNA polymerase α, Pol1p (Qi and Zakian 2000), and with telomere binding protein Stn1p, which is a primary effector of chromosome end protection (Pennock et al. 2001; Wang et al. 2000). Another telomere binding protein, Ten1p, was found to associate physically with Stn1p and it was able to suppress the mutant phenotype of *stn*1. Ten1p is also an essential protein as *ten*1-Δ is lethal. Like *stn*1 mutants, some *ten*1 mutants (*ten*1-16, -31) cause accumulation of single stranded DNA in telomeric regions of the chromosomes and they trigger DNA damage checkpoint and G2/M arrest, while others (*ten*1-3, -6, -13) cause telomere elongation, the expression of which is telomerase dependent (Grandin et al. 2001). Taken all together, it appears that the function of Cdc13p is to help recruit protein complexes, like Cdc13p-Stn1p-Ten1p, essential for both telomere length regulation and end protection (Grandin et al. 2001; Pennock et al. 2001).

7.3 Telomere Position Effect (TPE)

In addition to telomere length regulation and end protection of eukaryotic chromosomes, the telomere has yet another property, reminiscent of heterochromatin, genes adjacent to telomeres are transcriptionally repressed. This silencing effect is known as TPE, which is mediated by the SIR proteins, Sir2p, Sir3p, Sir4p (Bruin 2000; Laroche et al. 2000; Moretti and Shore 2001). The Sir2/3/4 protein complex is recruited to the chromosome sites by the telomere binding protein Rap1p that binds directly to the duplex telomere repeats. Experimental data showed that both Sir3p and Sir4p interact directly and independently with partially overlapping regions of the carboxyl terminus of Rap1p (Moretti and Shore 2001) and that the Sir protein complex interacts with the *N*-terminal tails of histones H3 and H4 in the subtelomeric nucleosomes (Hecht et al. 1995) where telomeric chromatin is hypoacetylated at each acetylatable histone H3/H4 lysine residue and this hypoacetylation is SIR dependent (Bruin 2000). Sir2p is shown to be a NAD-dependent histone deacetylase (Guarente 2000; Shore 2000) and hypoacetylation is directly correlated with transcriptional silencing (Bruin 2000). These authors also provided evidence that heterochromatin occludes the promoter, preventing its access to transcription factors. Cytologically, by double immunolocalization and FISH, Sir2/3/4p and subtelomeric sequences are found to colocalize in 6–10 perinuclear foci suggesting that these are sites of Sir-mediated silencing (Laroche et al. 2000). It is interesting to note that the telomere repeats are only ~300–400 bp from the chromosome end and yet Rap1p and Sir2/3/4 proteins were found at distances as far as 2–4 kb from a telomere end as shown by chromatin immunoprecipitation studies (Moretti and Shore 2001). This can be explained on the basis of telomere folding back or more likely on the basis of protein–protein cooperative bindings (Moretti and Shore 2001). Thus, the subtelomeric regions are heterochromatic and the heterochromatin can spread its influence on the adjacent genes, reminiscent of the classical variegated position effect found in Drosophila.

7.4 Telomeres Play a Role in Homologous Chromosome Pairing in Meiosis

Telomeres play an important role in homologous chromosome pairing during meiosis. Earlier, I speculated that telomeres attached to the nuclear envelope like casters rolling on a two dimensional surface in search for homologous contacts (Lu 1996). Now clear evidence has been documented (Niwa et al. 2000; Rockmill and Roeder 1998; Trelles-Sticken et al. 2000) and the best is provided by Trelles-Sticken et al. (2000) who used double immunolabeling of SPB components with fluorescein and of meiotic telomeres with antibodies against HA-tagged Ndj1p with rhodamine in synchronously induced meiosis in *S. cerevisiae* where Ndj1p has previously been identified as a meiosis specific telomeric protein (Chua and Roeder 1997). At early meiosis, telomeres were seen in peripheral rimlike distribution. This is followed by the so-called bouquet stage with telomeres clustering around the SPB, and finally at a later stage in pachytene showing dispersed telomere signals separate from the SPB. This clustering of telomeres is Ndj1p dependent as the bouquet formation is impaired in *ndj*1Δ mutants. This is the first telomeric protein identified to be required for meiosis specific bouquet formation (Trelles-Sticken et al. 2000). Here Ndj1p (also known as Tam1p) may be that caster I suggested

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previously. Telomere-mediated chromosome movements in meiotic prophase have also been suggested to facilitate homologous chromosome pairing by Yamamoto and Hiraoka (2001).

The bouquet formation has also been documented in *S. pombe* (Chikashige et al. 1997; Niwa et al. 2000), and it takes two steps to complete. First, in premeiotic haploid nuclei, centromeres are intimately associated with the SPB while telomeres are located at the opposite end of the nucleus. During the mating reaction, by the influence of the mating pheromone, telomeres become associated with the SPB. Second, centromeres detach from the SPB when conjugation and nuclear fusion are completed (Chikashige et al. 1997). Here it is evident that the interaction between the telomere and the SPB plays a key role in homologous pairing, and the telomere binding protein Taz1p of the fission yeast and SPB component Kms1p are required for telomere clustering and bouquet formation (Cooper et al. 1998; Niwa et al. 2000). The bouquet stage has been observed by cytologists in plants and animals for a long time, and it is only now that its significance has become known. By careful stage-by-stage analysis of telomere clustering in corn, Bass et al. (1997) showed that telomere clustering persisted through zygotene and early pachytene with the nucleolus located adjacent to the cluster. This could be the synizetic knot cytologists observed and never understood (Lu 1996).

8 CONCLUSIONS

Recent advances in FISH, fluorescence microscopy using fusion proteins (e.g., GFP) and indirect immunolabeling of epitope-tagged proteins in electron microscopy have made it possible to examine the cellular localization of DNA, RNA, and proteins of interest where localization may reveal structural organization or site(s) of action. Genetically, creation of temperature sensitive mutants by DNA manipulation and performing complementation with two hybrid system analysis have allowed in depth analyses of gene interactions as well as protein-to-protein interactions. Here the combination of molecular, cellular and genetic technologies has afforded a super powerful tool for life sciences research, some of which are high lighted in this chapter.

The biggest surprise is the finding that the nucleolus has varied functions not known before. Besides its known function for ribosome biosynthesis and its subcompartmentalization, it may be a transitory depot for mRNA export, it may hold keys to cell cycle and checkpoint control for mitosis and meiosis, and very importantly, it controls nucleolar silencing and aging.

The SPB is not just a nucleating site for spindle organization, it is intimately involved with mitosis exit network (MEN) as many of MEN components are localized to the SPB, and its position in the bud determines the time of exit from mitosis. The NPC is the gateway for nucleocytoplasmic trafficking. It is a very complex organelle. It includes ~30 nucleoporins (or nups) that are intimately associated with the protein imports and ribonuclear protein particle (RNP) exports. The cytoplasmic filaments and the nuclear basket are the docking sites for importin α and exportin β and their cargoes.

The centromere of the budding yeast is simple as compared to that of the fission yeast, but the kinetochore is a very complex organelle. It is responsible for connecting the spindle microtubules to both the old and the new SPB to ensure bi-orientation of sister chromatids at metaphase. The telomere may be considered an organelle. Telomere capping, silencing and end maintenance are vital to the life of the cell. Most importantly, telomeres play a key role in homologous chromosome pairing in meiosis.

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5

Genomics of Filamentous Fungi: A General Review

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1 INTRODUCTION

In recent years, we have witnessed a real revolution in the biological sciences with the advent of powerful technologies and applications. These new technologies have ushered biology into the "genomics era" where studies involving whole genomes are possible. In fact, the beauty of this technology is that it transcends the answering of old questions and challenges us to formulate and address even more complex biological questions. For example, the quality and the amount of data generated through the use of these techniques allow us to study not only the complex metabolic pathways in an individual, but through comparative genomics it also allows us to gain a better understanding of the evolution of development and metabolic pathways, and to determine the genetic relatedness among organisms.

In the following pages we tried to cover, as much as possible, recent advances achieved in the field, with an emphasis on work related to filamentous fungi. Due to the vast amount of data generated in recent years and the extensive use of the techniques and approaches described, we tried to be as inclusive as possible in covering the most recent work published at the time this manuscript was prepared.

2 FILAMENTOUS FUNGI

Fungi are a diverse group of organisms belonging to the Kingdom Mycota. They have been used through history in a multitude of industrial applications, and recently as a source of valuable enzymes. In the Far East, members of the genera *Aspergillus* and *Rhizopus* are used to produce miso, soy sauce, and tempeh, and different species of the genus *Penicillium* are used to ripen cheese. Throughout the world, common yeast is used in brewing and in baking; whereas mushrooms are cultivated and considered as a delicacy in many countries.

Fungi are also used for the production of many economically important products including antibiotics such as penicillin, enzymes such as amylases, proteinases, and rennin, and organic acids such as citric acid. The diverse metabolic activities of fungi have also been exploited in fields such as environmental remediation (Price et al. 2001). Moreover, the ability of certain species of fungi belonging to the genera *Candida* and *Yarrowia* to assimilate hydrocarbons is being investigated for potential usage in cleaning oil spills (Souciet et al. 2000). For a recent review about the importance of fungi please refer to the excellent work by Bennett (1998) as well as the following website http://www.cbs. knaw.nl/search_fdb.html.

The economical importance of fungi is not limited to their numerous uses in industry. Several fungi are considered to be human pathogens causing a wide scope of symptoms ranging from mere irritation in the case of athlete foot disease caused by *Trichophyton mentagrophytes* and *T. rubrum* to more serious illnesses which may lead to death as in the case of *Candida albicans*, *C. tropicalis*, *C. glabrata*, and *Cryptococcus neoformans*. The detrimental effects of these otherwise opportunistic fungi are especially observed with immunocompromised subjects (Souciet et al. 2000).

Several fungal species are also considered serious plant pathogens. In fact, Orke et al. (1994) estimated that worldwide losses in rice, wheat, potatoes, and maize incurred between 1988 and 1990 mounted to around US$ 64.6 billion and that 13% of the losses were caused by pathogens (Orke et al. 1994). Some fungal species are also known to produce metabolites on plants and prepared food that are toxic to animals and humans. Notable among these toxic fungal metabolites are the aflatoxins, fumonisins, trichothecenes, and ergot alkaloids (Richard and Payne 2002).

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Fungi have been studied extensively, not solely because of their economical importance, but also because of the very important biological questions that can be tackled by their use as model organisms. What makes fungi especially attractive for basic biological research is that these eukaryotes show differential spatial and temporal gene expression. Therefore, they are complex enough to use in studies on phenomena like differentiation and developmental processes, phenomena that cannot be studied to the same extent with simpler prokaryotes. Most fungal species are also easily maintained in culture. Moreover, extensive genetic studies, availability of well-characterized mutant strains, and the possibility of molecular manipulation with relative ease, have led to the usage of several filamentous fungi as model organisms. This is especially true in the case of *Aspergillus nidulans* and *Neurospora crassa*.

3 GENOMICS TECHNIQUES

The development of genomic sciences was dependent on significant innovations in several disciplines, including engineering, material sciences, and bioinformatics, that have made genome sequencing projects achievable. Novel technologies are developing in functional and structural genomics as well. Furthermore, new areas of investigation are now rendered both possible and necessary as a result of the vast amount of information derived from various genome-sequencing projects. Finally, all of the new techniques and applications are buttressed by the continuing development of robust computational tools that have allowed the organization and mining of the generated data.

3.1 Sequencing

3.1.1 Techniques

The basic approach for DNA sequencing, described several decades ago (Maxam and Gilbert 1977; Sanger et al. 1977) is based on the ability to determine the succession of the different bases forming a strand of DNA. The reaction requires, in addition to purified DNA, a DNA polymerase, a primer, and deoxynucleotides (dNTPs), the building units of DNA. The sequencing procedure described by Sanger, also known as the chain termination method, is basically a polymerase chain reaction (PCR) with the intention of synthesizing a DNA strand complementary to the strand to be sequenced (Mullis et al. 1986). However, contrary to a typical PCR reaction, dideoxynucleotides (ddNTPs) are added to the reaction. Upon the incorporation of a ddNTP, the extension of the synthesized DNA strand is stopped due to the lack of an available hydroxyl group necessary for attachment of the next dNTP. The addition of the ddNTPs results in the generation of a multitude of DNA fragments of different size, complementary to the DNA strand to be sequenced. The different fragments have identical $5'$ ends corresponding to the $5'$ end of the primer used in the sequencing reaction. Their $3'$ ends however, are different, depending on the particular point at which the extension reaction was terminated as a result of the addition of a particular ddNTP. The generated fragments are then separated by electrophoresis, a technique based on the pioneering work by Tiselius on the separation of proteins in 1937 (Fitch and Sokhansanj 2000). The sequence of the target DNA can be inferred from the separation pattern of the different generated fragments on the separating gel, judging from the size of the fragments and the identity of the ddNTP that resulted in their production.

Although simple in principle, the sequencing reaction can be cumbersome and time consuming since it involves many steps that need to be optimized including the extraction, cleaning, and handling of the DNA to be sequenced, the design and synthesis of the primer to be used in the sequencing reaction, the synthesis of high quality dNTPs and ddNTPs, and the production of high fidelity DNA polymerase. Also of importance is the availability of a robust electrophoresis system that provides adequate separation of the generated DNA fragments in a reliable and reproducible manner. Furthermore, to indulge in the sequencing of entire genomes, there was an imposing need to automate as many of the previously described steps as possible to make the whole procedure amenable to transformation into a high throughput operation.

Several advances in the chemistry of the sequencing reaction have led to cheaper, more reliable, and more reproducible sequencing. These include advances in the production of better DNA polymerases providing longer more consistent sequences, and progress in the mass synthesis of cheap oligonucleotides to satisfy the demands of sequencing projects. The MerMade synthesizer developed at the University of Texas Southwestern Medical Center (Rayner et al. 1998) is an example of the many oligonucleotide synthesizers available in the market. It is capable of producing two 96-well plates of oligonucleotides every 17 h (Meldrum 2000a, b). Perhaps, one of the most important advances in the chemistry of the sequencing reaction is the use of fluorescent dyes to tag the various fragments generated. Two different techniques have been used; the dye terminator method and the dye primer method (Meldrum 2000a). In the dye terminator method the ddNTPs are tagged with different dyes that fluoresce upon excitation by a laser at specific wavelengths (Hunkapiller et al. 1991; Smith et al. 1985; Smith et al. 1986). In the dye primer method, the sequence-specific primer is labeled instead of the ddNTPs, and since the ddNTPs are not tagged, the sequencing reactions with the different ddNTPs should be performed separately in four different aliquots. In the dye terminator method however, all four ddNTPs are usually included in the same reaction. This makes dye termination the method of choice in most sequencing projects (Fitch and Sokhansanj 2000). Tagging the produced fragments was necessary to automate the process of "base calling," the automatic determination of the sequence of the target DNA from the sequential determination of the subsequent ddNTPs which incorporation leads to the termination of the sequencing reaction. The automated base

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calling is coupled to the separation of the generated fragments by electrophoresis.

Innovations in electrophoresis were also instrumental in the development of the modern DNA sequencing machines that transformed sequencing into a high throughput and highly automated process. The shift from slab-gel-based (where the DNA separating material, such as polyacrylamide, is spread thinly between two glass plates) to capillary-based instrumentation, (where the sieving matrix is injected into capillaries) was central in the development of machines such as the ABI PRISM 3700 DNA Sequencer from PE Biosystems (Applied Bio-systems) and the MegaBACE 1000 from MolecularDynamics (Amersham-Pharmacia Biotech); the two main workhorses used in the major DNA sequencing centers (Mitnik et al. 2001). Both of these instruments can process 96 samples per run (usually two hours) with a resolution of a single base starting from as little as 20 ng of DNA per sample (Fitch and Sokhansanj 2000). The automated sequencers are also equipped with detection mechanisms to excite and detect the dyes used in the sequencing reactions. Different machines use different optics to detect the dyes though the fluorescence is usually induced with an Argon ion laser (Fitch and Sokhansanj 2000). For a more detailed exposition of the techniques used in DNA sequencing, please refer to these excellent reviews: (Esch 2000; Fitch and Sokhansanj 2000; Green 2001; Meldrum 2000a,b; Righetti et al. 2002).

3.1.2 Sequencing Strategies

In spite of the tremendous advances in DNA sequencing technologies, there still exist serious limitations, especially *vis-à-vis* the length of read that can be obtained from a single sequencing reaction. Currently, such technical limitations restrict the size of a single read to around 500–800 bases (Righetti et al. 2002). This has presented a challenge to genome sequencing projects, since determining the sequence of a genome requires the assembly of thousands of reads. While this problem has not been solved, several techniques have been developed to increase sequencing efficiency. One of the most important advances is the automation of DNA sample preparation to the extent that most of the steps involved in this process are now automated. These include the use of sophisticated plaque and colony pickers, which have decreased the costs and the time required to carry out large amount of library handling and subcloning needed in sequencing projects. Other steps that have been automated include the purification of the subcloned fragments of the template DNA from bacteria, the preparation of the sequencing reactions, and even the application of the sequencing reactions to the sequencers (Righetti et al. 2002). Generation of large amount of sequences also required the development of software to "call" the different bases resulting from the sequencing reactions and to assemble the different fragments in adjoining contigs. The codes Phred, Phrap, and Consed were developed by Phil Green's group at the University of Washington (Ewing and Green 1998; Ewing et al. 1998; http://www.phrap.org/; http://www.phrap.com/phred/), and are widely used for base calling, contigs assembly, and viewing of sequence assemblies respectively.

Several strategies were devised to tackle the sequencing of full genomes, in an attempt to complete the different sequencing projects in the most cost effective way and in the least amount of time. For a comprehensive review of the different strategies adopted in genome sequencing projects, we refer you to the review article by Green (2001). Most sequencing strategies used currently are based on shotgun cloning (Anderson 1981; Gardner et al. 1981). This strategy consists of shearing the genomic DNA into smaller pieces either chemically or mechanically. The sheared fragments are subsequently cloned and sequenced. There are two main approaches in this strategy. The first involves shearing the genomic DNA into relatively large pieces (usually around 200 kb) and cloning them into a yeast artificial chromosome (YAC) or, more commonly, into a bacterial artificial chromosome (BAC) to create a library. In this mapping approach, the cloned fragments are then assembled into maps (Fitch and Sokhansanj 2000). Selected BACs are then fragmented into smaller pieces (around 2 kb), subcloned in plasmids, and sequenced. Generated sequences are called and assembled automatically using computational tools such as Phred and Phrap. The assembly is then completed using the information derived from the rough maps that were previously generated. An alternative strategy to the mapping approach is the "whole-genome shotgun approach" (Green 2001). In this approach the genomic DNA is directly sheared into small fragments (2 kb), and cloned into bacterial plasmids. Fragments are then sequenced and assembled using rigorous computational tools. The main advantage of this approach is avoiding the mapping step, the making of a BAC library and the subsequent subcloning steps. The main drawback of this approach however, is that the final assembly of the sequences may present a challenge in the absence of a clone-based physical map. This is especially true in the case of larger and more complex genomes due to the huge number of generated fragments and the presence of a large number of redundant repeats.

3.2 Functional Genomics

Deciphering the enormous amount of DNA sequence data produced by various genome projects created the need to devise new techniques to identify different genes and to characterize them by determining their functions. Different approaches are currently used for this purpose. The ultimate goal has always been to discern the activity of genes by identifying and studying their products, i.e., the proteins they encode (proteomics), or the final enzymatic reactions that they control (metabolomics). This approach should ultimately elucidate the intricacies of the ways organisms interact and respond to the various cues they receive from their environment.

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Another approach is to concentrate on the expression levels of the genes, i.e., the abundance of the mRNA they encode under different conditions. Gene expression profiling as a means to study the functions of different genes has been the choice of many researchers, resulting from the availability of many techniques that are amenable to this purpose. We will cover in this section the most recent technologies used in gene expression profiling. Advances in proteomics and metabolomics will be covered later in the chapter.

Several techniques have been used for many decades to study the pattern and level of gene expression by directly measuring the fluctuations in the levels of mRNA accumulation under various conditions. Northern transfer hybridization with its variations, *in situ*-hybridization techniques, S1 nuclease analysis, and more recently reverse transcriptase PCR (RT-PCR), are only a few examples. These techniques, however, are not amenable to high throughput analysis due to many technical limitations. New approaches had to be devised based on emerging technologies. One main theme underlying all these technologies is, in addition to their amenability to automation and thus suitability for high throughput type operations, their ability to provide a holistic view of the expression patterns of a multitude of genes simultaneously as opposed to the one-gene at a time approach that has prevailed because of the limitations of previous techniques (Donson et al. 2002). Many techniques have been devised for this purpose. We will describe some of those that are most commonly used, mainly large-scale expressed sequence tag (EST) sequencing, Serial Analysis of Gene Expression (SAGE), differential display, and microarray analysis.

3.2.1 Large-Scale EST Sequencing

This technique is based on generating complementary DNA from a population of mRNA extracted from the tissue of interest. The produced cDNAs are subsequently cloned and sequenced. Expression profiling is then deduced from the relative abundance of the different sequences with the idea being that the level of expression of a certain gene will be reflected by the level of its transcribed mRNA, and hence by the abundance of the corresponding cDNA in the analyzed clones as revealed by sequencing. This technique provides a way to directly estimate the level of expression of a certain gene or a set of genes in a quasi-quantitative way. Furthermore, this technique is amenable to high-throughput analysis. The major drawback is the extensive amount of sequencing needed in such a project to be able to draw statistically valid conclusions, given the technical limitations of this technique. For a more thorough discussion of EST analysis, refer to Bohnert et al. (2001) and Ohlrogge and Benning (2000).

3.2.2 Serial Analysis of Gene Expression

This technique developed by Velculescu (1995) is comprised of three steps. The first step is the generation of "sequence tags," that is a population of short oligonucleotides (10–14 bp) specific to the different cDNA strands prepared from mRNA collected from the tissue of interest. The tags are then concatenated, cloned, and sequenced. Subsequently, the different clones are sequenced and submitted to rigorous computational analysis. The patterns of expression (profiles) of the different genes are inferred from the abundance of the different tags. This technique can be used to do expression profiling starting from a small amount of tissue. It does reduce the amount of sequencing needed compared to EST analysis, but it requires extensive computational power and is most suited to organisms with available extensive cDNA sequence database (Donson et al. 2002). The website http://www.sagenet.org/ is an excellent source of information on SAGE and includes protocols, publications, and major projects using the technique.

3.2.3 Differential Display

A set of methodologies has been devised to study expression by direct analysis of the corresponding cDNA. Differential display is one of these methods that have been popular (Liang and Pardee 1992). In this approach, the mRNA of interest is collected and submitted to reverse transcription. The produced cDNA is then amplified with PCR. This is followed by the separation of produced products on a separating matrix. Banding profiles from cDNA obtained from different mRNA populations are compared. Interesting bands, usually ones that are showing polymorphism in presence or in intensity are extracted from the gel and amplified for further characterization such as sequencing. The different variations on this technique as well as its various applications have been covered by in an excellent review by Matz and Lukyanov (1998). Differential display does, however, bear many limitations and though it has been very useful in gene discovery, its usefulness in gene expression profiling and analysis has often been questioned because of its heavy reliance on PCR and the relatively high occurrence of false positives upon its usage (Donson et al. 2002).

3.2.4 Microarray and Chip Analysis

Microarray analysis is gaining increasing popularity among researchers interested in gene expression profiling as is evidenced by published data generated by this technique. Technical improvements and innovations that rendered microarray analysis more reliable and cost effective are making this technique widely used by an ever-increasing number of researchers from different disciplines. Examples of studies using microarray analysis will be covered in the next section of this chapter.

The technique is a variation of Northern analysis that was first described by Alwine et al. (1977) and had been used since as one of the main techniques of determining the level of expression of a certain gene by monitoring the levels of the corresponding mRNA produced by the cell machinery under certain well-defined conditions. Like Northern analysis,

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microarray analysis is a hybridization-based approach (Donson et al. 2002), but contrary to the former technique, it provides the ability to monitor the expression of thousands of genes in parallel, which makes it ideal for gene profiling in a genomics context.

Microarray analysis can be divided into the following steps: Preparation of the probes to be used in the analysis, application of these probes to an appropriate matrix in arrays, fixing the arrayed material, preparation and labelling of the material to be hybridized to the array, hybridization, data collection, data analysis, and data storage (Blohm and Guiseppi-Elie 2001).

The most popular arrays used, fall into two major categories: oligonucleotide arrays and cDNA arrays. In oligonucleotide arrays, such as the GeneChips arrays produced by Affymetrix (http://www.affymetrix.com/; Lockhart and Winzeler 2000), specific oligonucleotides, representative of the genes to be profiled, are directly synthesized on a solid support. Oligonucleotide arrays for several organisms such as yeast, arabidopsis, and maize are available (Donson et al. 2002) and several companies will manufacture tailored arrays on demand. The technology used to produce these arrays is, however, still beyond the abilities of most laboratories. The different oligos are synthesized using photolithographic techniques originally developed for the manufacturing of semiconductors (Fitch and Sokhansanj 2000; Fodor et al. 1991; Van Hal et al. 2000). The specific oligonucleotides are produced by the successive application of photoprotected nucleotides on a glass surface. A photomask is then used to selectively illuminate specific locations of the glass, activating specific nucleotides and rendering them capable of coupling. The subsequent applications of photoprotected nucleotides and their selective activation with photomasks, ensures the synthesis of the desired oligos (Chee et al. 1996; McGall et al. 1996). Obviously, this requires a high degree of sophistication in terms of automation and computational abilities to manufacture reliable DNA chips.

In cDNA arrays pioneered by Patrick Brown's lab at Stanford (http://cmgm.stanford edu/pbrown/mguide/index.html; Schena et al. 1995), the probes to be spotted are usually amplified by PCR from templates such as EST libraries (Donson et al. 2002). The PCR products are then cleaned and spotted on coated glass slides. Different chemicals are used to coat the slides with the aim of optimizing the binding of the applied DNA and reducing the noise ensuing from nonspecific hybridizations. Slides with amine, aldehyde, or polylysine chemistries are most commonly used. Slides are available from several manufacturers (http://www.arrayit.com/,http://www.cmt.corning.com/) or can be manufactured in house (http://cmgm.stanford.edu/pbrown/protocols.html). The arraying is automated, and arraying robots can also be acquired from several manufacturers. For reviews covering the different aspects of arraying technologies refer to (Blohm and Guiseppi-Elie 2001; Hegde et al. 2000).

Prepared arrays are hybridized with labeled cDNA produced from mRNA extracted from the tissues to be analyzed. The cDNA is labeled with fluorescent dyes; the fluorescent cyanine dyes Cy3 and Cy5 are currently the most popular dyes used. These two chromophors fluoresce at 633 nm for Cy5 and 543 nm for Cy3. One of the highly attractive qualities of microarray analysis is the ability to analyze the expression profile from two different tissues simultaneously using the same array. This is achievable by labeling the cDNA from the two different tissues with either Cy3 or Cy5. After hybridization, the arrays are scanned usually using a confocal laser scanner, and the fluorescence intensities of the two different dyes are measured. The data are then analyzed by different software to determine the levels of expression of the different genes as deduced from the relative fluorescence of the corresponding probes. Different approaches are used to analyze the thus produced data ranging from simple paired t-tests (Baldi and Long 2001) to more complicated analysis involving analysis of variance (ANOVA) models (Kerr et al. 2000; Wolfinger et al. 2001). Nevertheless, it was shown that this technique can be used to detect differences of expression in the range of two fold or less (Blohm and Guiseppi-Elie 2001) with reported sensitivities up to one copy in 300,000 transcripts (Lockhart et al. 1996). The large amount of data generated from microarray experiments require the development and maintenance of advanced laboratory information management systems (LIMS) where the different stages of the experiment can be traced, the different used materials tracked, and the produced data stored in relational databases (Bassett et al. 1999; Ermolaeva et al. 1998). In Table 1, we display a number of hyperlinks to resources on the web covering different aspects of microarray analysis.

3.3 Computational Genomics Tools

Most of the techniques described previously require considerable computational power at different experimental stages, including data generation, data collection, and data analysis. We will restrict the discussion in this section to available computational genomics tools, that is tools developed to handle the analysis of sequencing data (Tsoka and Ouzounis 2000). Table 2 is a compilation of some of these tools. Computational genomics is the branch of computational biology that deals with the analysis of entire genome sequences (Tsoka and Ouzounis 2000). The first step after acquiring sequence data is often the submittal of these data to a database. The availability and the accessibility of such databases by the public of researchers have proved to be instrumental in many respects. Most importantly, these databases necessitated the production of standardized protocols and formats for data deposition, storage, and retrieval. Some of the major databases include the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/ and the European Molecular Biology Laboratory (EMBL) http://www.embl-heidelberg.de/, two

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Table 1 Microarray resources on the World Wide Web

Microarray resources	
Protocols	
http://cmgm.stanford.edu/pbrown/protocols/index.html	Stanford University
http://www.tigr.org/tdb/microarray	The Institute for Genomic Research
Providers of slides	
http://www.cmt.corning.com/	Corning
http://www.arrayit.com/	Telechem
Providers of spotting robots	
http://www.cartesiantech.com/	Cartesian Technologies
http://www.packardinst.com/	Packard
http://www.genomicsolutions.com/	Genomic Solutions
http://www.genetix.co.uk/	Genetix
Providers of scanners	
http://www.gsilumonics.com/	GSI Lumonics
http://www.geneticmicro.com/	Genetic Microsystems
http://www.genomicsolutions.com/	Genomic Solutions
http://www.mdyn.com/	Molecular dynamics
Image analysis	
http://experimental.act.cmis.csiro.au/Spot/index.php	CSIRO Mathematical and Information Sciences, Image Analysis Group
http://www.biodiscovery.com/	BioDiscovery
http://imaging.brocku.ca/Arrayvision.html	Imaging Research
http://rana.lbl.gov/EisenSoftware.htm	Stanford University
Data analysis	
http://www.biodiscovery.com/	BioDiscovery
http://www.sigenetics.com/	Silicon Genetics
http://www.spotfire.com/	Spotfire
http://rana.stanford.edu/software/	Stanford University
http://www.tigr.org/softlab/	The Institute for Genomic Research
http://www.stat.berkeley.edu/users/terry/zarray/Html/index.html	Terry Speed's Group
http://www.ncbi.nlm.nih.gov/geo/	National Center for Biotechnology Information
http://www.ncgr.org/genex/	National Center for Genome Resources
http://www.jax.org/research/churchill/software/anova/index.html	Gary Churchill's Group

excellent resources for gene annotation, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) http://www.genome.ad.jp/kegg/, which is mainly a database of metabolic pathways, and Pfam http://pfam.wustl.edu/index.html, a protein domain database from Washington University (Skinner et al. 2001).

Recent advances in computational genomics were instrumental in the development of tools to perform a wide array of tasks ranging from genome sequence analysis, including gene coding, database searching, and functional annotation, to more sophisticated operations such as dissecting metabolic pathways and signaling networks, distinguishing functional classes, and identifying phylogenetic patterns (Tsoka and Ouzounis 2000). Perhaps one of the most significant advances in this area was the adoption of Hidden Markov Models (HMM) (Eddy 1996) for the development of a series of tools used for sequence analysis. The utilization of HMM allowed the development of a whole generation of algorithms able to transcend the limitations of the tools previously used for pair wise sequence comparisons. The new tools are more amenable to training, providing much needed flexibility in performing sequence comparisons. Such flexibility is very important, especially if the aim of the sequence analysis is to go beyond the direct pair wise comparison and to discern nonobvious similarities that may be very indicative of conserved residues and domains. These tools, in fact, provided a better framework to set position-specific residue scores, score gaps and insertions, and to combine structural and multiple sequence information for the generation of profile methods used in protein structure prediction and large-scale genome sequence analysis. Following is a brief description of some of the available analytical tools, our aim being to present the reader with a representative sample of these tools and to highlight their vital role in the success of genomics projects.

One important aspect of sequence analysis is the ability to determine Open Reading Frames (ORFs) and thus distinguish coding regions in the DNA sequence from noncoding regions.

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Table 2 Available computational tools

Computational tool	Web address
Gene coding	
Glimmer	http://www.tigr.org/software/glimmer/
GenMark	http://www.ebi.ac.uk/genemark/
Database searching	
LAMA	http://blocks.fhcrc.org/blocks-bin/LAMA_search
Hmmer	http://analysis.molbiol.ox.ac.uk/pise_html/hmmemit.html
MAST	http://bioweb.pasteur.fr/seqanal/motif/meme/mast-databases.html
SAM-T99	http://www.cse.ucsc.edu/research/compbio/sam.html
Sequence clustering	
Pfam	http://pfam.wustl.edu/
COGS	http://www.ncbi.nlm.nih.gov/COG/cognitor.html
WIT	http://www-unix.mcs.anl.gov/compbio/
Protomap	http://www.protomap.cs.huji.ac.il/new_seq.html
Emotif	http://motif.stanford.edu/emotif/emotif-scan.html
Functional annotation	
EcoCyc	http://www.ecocyc.org/
SGD	http://genome-www.stanford.edu/Saccharomyces/
FlyBase	http://flybase.bio.indiana.edu/

This is not a trivial task and requires, in addition to the development of proper algorithms, the availability of extensive databases to train these algorithms into tools sensitive enough to identify ORFs. GeneMark; (http://www.ebi.ac.uk/genemark/) and Glimmer (Gene Locator and Interpolated Markov Modeler) (http://www.tigr.org/software/glimmer/) are two examples of DNA coding region identifying tools.

Several tools were developed to perform database searching. Local Alignment of Multiple Alignments (LAMA) (http://blocks.fhcrc.org/blocks-bin/LAMA_search; Henikoff et al. 1999) is a sensitive method designed to detect relatively weak sequence similarities between conserved regions of protein families. It functions by comparing a DNA or a protein sequence to a database of protein blocks. Alternative HMM-based methods include, Hmmer developed at the Pasteur Institute (http://analysis.molbiol.ox.ac.uk/pise_html/hmmemit.html; Bateman et al. 1999) SAM-T99 (Sequence Alignment and Modeling System) (http://www.cse.ucsc.edu/research/compbio/sam.html) developed at University of California, Santa Cruz, and MAST (Motif Alignment and Search Tools) (http://bioweb.pasteur.fr/seqanal/motif/meme/mast-databases.html; Bailey and Gribskov 1998). Pfam, Cluster of Orthologous Groups of proteins (COGS) , What Is There? (WIT), Protomap, and Emotif are a set of programs used for clustering of genes and proteins into families (Tsoka and Ouzounis 2000). Pfam (http://pfam.wustl.edu/; Bateman et al. 2000) is a database of multiple alignments of protein domains. It is very useful in determining whether a new protein sequence can be clustered with an existing protein family, particularly when dealing with multidomain proteins. The COGS (http://www.ncbi.nlm.nih.gov/COG/cognitor.html; Tatusov et al. 1997) is a similar database built using protein data derived from 43 complete genomes. The WIT (http://www-unix.mcs.anl.gov/compbio/; Overbeek et al. 2000) contains data from 38 sequenced genomes, providing yet another useful tool for protein alignments and clustering in addition to mining for functional domains. Protomap (http://www.protomap.cs.huji.ac.il/new_seq.html; Yona et al. 1999) is yet another clustering tool using a database founded on the classification of all the proteins in the SwissProt (http://expasy.hcuge.ch/) database into biological families and superfamilies. Finally, Emotif (http://motif.stanford.edu/emotif/emotif-scan.html; Nevill-Manning et al. 1998) is another clustering program developed at Stanford University.

EcoCyc, SGD, and FlyBase, offer tools for functional annotation. They provide a reference source where individual sequences are linked to different types of information such as accession numbers, related domains, assigned functions of related proteins, enzyme commission (EC) numbers, relevant biochemical pathways, and even links to selected literature (Tsoka and Ouzounis 2000). This is instrumental when a system-level understanding of a certain organism is sought. These types of databases are usually designed to be an exhaustive source of information about a particular organism. EcoCyc (http://www.ecocyc.org/; Karp et al. 2000) is mainly an *Escherichia coli* database resource, and SGD (http://genome-www.stanford.edu/ Saccharomyces/; Gelbart et al. 1997) is a *Saccharomyces cerevisiae* database, whereas FlyBase (http://flybase.bio.indiana.edu/; Cherry et al. 1998), a joint project with the Berkeley Drosophila Genome Project, is a comprehensive resource for information on *Drosophila melanogaster*.

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Table 3 Major fungal sequencing projects

Organism	Sequencing center(s)	Web link
A. fumigatus	The Sanger Center, University of Manchester, Pasteur Institute, TIGR	http://www.sanger.ac.uk/Projects/A_fumigatus/
A. nidulans	Cereon genomics	http://microbial.cereon.com/
C. albicans	Stanford DNA Sequencing and Technology Center	http://sequence-www.stanford.edu/group/candida/index.html
C. neoformans	British Columbia Genome Sequence center, Nagasaki University School of medicine, University of Oklahoma, Stanford Genome Technology Center	http://www-sequence.stanford.edu/group/C.neoformans/index.html
P. chrysoporium	The DOE Joint Genome Institute	http://www.jgi.doe.gov/programs/whiterot.htm
M. grisea	The Whitehead Institute for Genome Research, North Carolina State University	http://www.fungalgenomics.ncsu.edu/index.htm
N. crassa	The Whitehead Institute Center for Genome Research	http://www-genome.wi.mit.edu/http://www-genome.wi.mit.edu/
P. carinii	The Sanger Institute	http://www.sanger.ac.uk/Projects/P_carinii/
U. maydis	LION Bioscience and Bayer	Not available for the public
S. cerevisiae	Stanford DNA Sequencing and Technology Center	http://genome-www.stanford.edu/Saccharomyces/
S. pombe	The Sanger Institute	http://www.sanger.ac.uk/Projects/S_pombe/

4 RECENT ADVANCES

4.1 Sequencing Projects/Available Genome Sequences

Recently, there has been an overwhelming amount of sequencing data produced by both public and private institutions, and more projects are being initiated. A landmark event that boosted interest in sequencing projects was, undoubtedly, the announcement on June 25, 2000 of the completion of a working draft sequence of the human genome (http://www.ornl.gov/hgmis/project/clinton1.html) and the publication that followed (Venter et al. 2001). Despite the fact that the first full determined genome sequence of a eukaryotic organism was that of the fungus *S. cerevisiae* in 1996 (http://genome-www.stanford.edu/Saccharomyces; Mewes et al. 1997), a relatively small number of fungal genomes have been or are being sequenced. In fact, from around 800 organisms whose genome can be found in the databases of the NCBI (July, 2002) (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = Genome) including organisms belonging to bacteria, archae, and eukaryota along with many viruses and organelles, only eight are fungi. These are *A. fumigatus*, *A. nidulans*, *C. albicans*, *Cryptococccus neoformans*, *N. crassa*, *Phanerochaete chrysoporium*, *S. cerevisiae*, and *Schizosaccharomyces pombe*. In Table 3, we list the major sequencing projects involving fungi at the time this manuscript was prepared.

4.2 Relevance of Sequencing Data

The data produced by these sequencing projects are instrumental for the development of studies involving other fields of genomics such as functional genomics or metabolomics. The sequencing data itself also has been useful in answering several important questions in fields such as epidemiology and evolution. An example of this is the work by Braun et al. (2000). They compared sequence data from the filamentous fungus *N. crassa* and the yeast *S. cerevisiae* and discovered that *N. crassa* has a higher proportion of "orphan" genes, genes with no identified homologs in the database, in comparison with *S. cerevisiae,* orphan (Braun et al. 2000). The researchers tried subsequently to address the greater complexity of the genome of *N. crassa* compared to that of *S. cerevisiae* and to produce a theory explaining this complexity. To do this they compared sequences of genes from the two fungi to sequences of homologous genes in the database. From this study they concluded that the greater complexity of *N. crassa* resulted from the acquisition by the fungus of novel genes, and that some of this acquisition could be attributed to horizontal gene transfer from prokaryotes as was previously proposed (Prade et al. 1997). They also concluded that there is no evidence of an increased rate in evolution between the two fungi, which would have been an alternative explanation for the higher complexity of *N. crassa*. Interestingly, they found that

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S. cerevisiae has lost some genes during its evolution, which concurs with the theory that budding yeast, a unicellular fungus, is actually derived from a multicellular ancestor (Berbee and Taylor 1993; Liu et al. 1999).

Another example of the type of information that can be inferred from comparative genomics is illustrated in findings of the Génolevures project (http://cbi.labri.u-bordeaux.fr/Genolevures/Page_intro.php3). This project involves comparing *S. cerevisiae* to 13 other yeast species. In a series of articles in FEBS Letters, the group published their findings regarding a set of topics including synteny (Llorente et al. 2000), genetic redundancy (Blandin et al. 2000), functional classification of genes (Gaillardin et al. 2000), genome evolution, and speciation where they discovered that gene sequence drift was one of the main determinants of speciation in hemiascomycetous yeasts (Souciet et al. 2000).

The deposition in the various databases of additional fungal sequences generated by the different sequencing projects will lead to more extensive comparative studies involving filamentous fungi. This will be instrumental in elucidating some issues such as determining the mechanisms of recombination in fungi and their frequencies, uncovering the patterns of genetic transmission, and identifying some of the adaptive mutations in fungi which may have great implications in terms of isolating elements affecting pathogenicity, virulence, drug resistance, and enhanced fitness in fungi (Anderson and Kohn 1998).

4.3 Relevance of Data Derived from Functional Studies

Gene expression studies are a very important component in projects aiming for a complete characterization of the gene set identified in an organism. As we discussed earlier, several techniques have been developed to determine gene expression profiles at a genomic level; that is to analyze the expression patterns of a multitude of genes in parallel.

Several groups relied on the analysis of ESTs to analyze patterns of gene expression. Keon et al. (2000) used this approach with the plant pathogen *Mycosphaerella graminicola* (*Septoria tritici*) the causal agent of a leaf blotch on wheat. The ESTs were derived from a cDNA library built from cells that had been transferred from a medium where nitrate was the sole nitrogen source to one where the main nitrogen source was ammonium. The rationale being that the nutrient shift would result in the acidification of the medium, which would lead to the induction of genes involved in virulence (Keon et al. 2000). This acidification of the medium is believed to mimic the prevailing conditions in the host upon infection and colonization by the fungus. Their study led to the identification of 704 unique ESTs with various potential functions ranging from primary metabolism and signal transduction to cell structure and cell division (Keon et al. 2000). Kamoun et al. (1999) utilized the same type of analysis with the oomycete *Phytophthora infestans*, the causal agent of the famous potato late blight that lead to a devastating famine in Ireland in the late 19th century. In this project, the gene diversity of *P. infestans* was assessed through the analysis of ESTs obtained from a cDNA library constructed from mycelial RNA. 760 unique genes were identified including four novel genes believed to code for elicitin-like proteins. Elicitins are a family of structurally related proteins shown to induce the hypersensitive response upon the infection of *Nicotiana* plants by *P. infestans* (Keon et al. 2000). Furthermore, Thomas et al. (2001) used EST analysis to identify genes in *Blumeria garminis*, a plant pathogen that causes powdery mildew on barley, using cDNA libraries constructed from RNA obtained from ungerminated and germinated conidia. In an attempt to construct a molecular database of early development of this obligate pathogenic fungus, 4908 ESTs were analyzed. The study led to several interesting observations including some conflicting evidence of a role of a calcium signaling pathway and a cAMP-regulated signaling pathway in the regulation of the germ tube emergence and appressorium differentiation in *B. graminis* (Thomas et al. 2001).

The SAGE was also used to study gene expression patterns. In an analysis of global gene expression in *S. cerevisiae*, Basrai et al. (1999) identified 302 previously unidentified transcripts from nonannotated open reading frames (NORF). Further analysis of one of these NORFs, HUG1, revealed that this gene is a component of the MEC1-mediated checkpoint response to DNA damage and replication arrest in *S. cerevisiae* (Basrai et al. 1999). Kal et al. (1999) used SAGE to compare gene expression profiles from yeast grown on two different carbon sources, the fatty acid oleate and glucose. They were able to document that the fungus adapted to the change in carbon source through an increase in the production of mRNAs encoding peroxisomal β-oxidation enzymes. Their study also revealed the existence of redox shuttles across organellar membranes involving peroxisomal, cytoplasmic, and mitochondrial enzymes (Kal et al. 1999).

The use of microarrays is yet another very popular way to study gene expression. One good example of the power of functional genomics in general, and microarray analysis in particular, is the work by Hughes et al. (2000) on the annotation of genes from yeast. A compendium approach was adopted in which the transcript profiles of 4553 genes were characterized in 300 different mutants, under different physiological situations and following various chemical treatments. The compendium provided a wealth of information, including the assignment of putative functions to several ORFs including involvement in ergosterol biosynthesis, cell-wall structure, and mitochondrial function. Their data were also instrumental in discovering that the anesthetic drug dyclonine affects the yeast gene *erg2p*, which lead to the identification of the cellular target of the drug in humans and turned out to be the neuroactive sigma factor that shows a great sequence homology to *erg2p* (Hughes et al. 2000). These results illustrate the strength of these techniques especially in systems where a large number of mutants are

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available and where results from functional genomics projects can be coupled to existing genetic studies and characterizations.

The availability of large annotated gene mutant collections can be instrumental to fully exploit the information derived from sequencing and functional analysis data. Such collections are now available in several model organisms including yeast (Winzeler et al. 1999), *Arabidopsis* (Hamer et al. 2001b) and mice (Metzger and Feil 1999). The ability to make such libraries is however contingent on the availability of an efficient gene disruption system that would be amenable to perform high throughput gene disruptions. The development of such a system for many filamentous fungi is still problematic because of the lack of proper transformation systems or due to the relatively low rate of targeted integrations during transformations (Asch and Kinsey 1990). Recently, a group from Paradigm Genetics introduced a new strategy for gene discovery and function assignment in filamentous fungi (Hamer et al. 2001a). They termed the strategy (TAGKO) for Transposon-Arrayed Gene Knockouts. The method is based on using transposons to mutagenize cosmids *in vivo*. The transposons can then be used to tag genes for sequencing, and the mutagenized cosmids can subsequently be used as disruption vectors. This approach was used to investigate an amino acid oxidation pathway in the two important plant pathogens, *Magnaporthe grisea* and *Mycosphaerella graminicola* (Hamer et al. 2001a).

5 EMERGING TECHNOLOGIES

Many of the techniques used in functional genomics rely on measuring the mRNA as a means to assess the levels of gene expression. New techniques are being developed to investigate other aspects of gene expression such as the analysis of the types of proteins produced and their profiles, or the detection and identification of the different types of metabolites produced by an organism and their fluctuations under different gene expression patterns. The combined usage of these different approaches would undoubtedly provide a better understanding of the intricacies of gene expression.

5.1 Proteomics

Proteomics involves the high-throughput simultaneous study of proteins in a cell (Esch 2000). The basic technique used in proteomics is two-dimensional gel electrophoresis (Quadroni and James 1999) in which proteins are applied on a matrix and separated under the influence of an electric field. The patterns and the intensities of the resolved spots representing the different proteins present in the sample are then analyzed and compared to patterns collected under other conditions. The coupling of 2-D gel electrophoresis to mass spectrometry enhanced the efficacy of the technique by providing a means for a high-throughput identification of the separated proteins (Fitch and Sokhansanj 2000). Two main spectroscopic approaches are being used in proteomics, tandem mass spectrometry, and matrix-assisted laser desorption/ionization (MALDI).

In tandem mass spectrometry, the different peptides are digested with a set of proteases. The mass of the resulting peptidic fragments is determined spectroscopically and is used to assign a characteristic fragmentation profile for the protein. The protein is subsequently identified by comparing its fragmentation profile to profiles available in the databases (Quadroni and James 1999). In, MALDI, instead of sequencing individual peptides, the mass spectrum of the eluted peptide mixture is determined. The protein is then identified by comparing its peptide-mass spectrum to other spectra in the database (Henzel et al. 1993). Pardo et al. (2000) used a proteomic approach to study *S. cerevisiae* cell wall biogenesis. Using a combination of 2-D electrophoresis and mass spectrometry, they attempted to analyze proteins secreted by regenerating protoplast. They were able to identify 32 different proteins that are potentially involved in cell wall biosynthesis, some of which were completely novel (Pardo et al. 2000).

Proteomic studies will ultimately go beyond the simple characterization of protein profiles to more intricate protein analysis, investigating areas such as posttranslational modifications and protein–protein interactions (Pandey and Mann 2000). A myriad of technologies are being developed to perform such studies.

One approach has been to develop protein arrays to perform studies ranging from analysis of protein interactions to studying enzymatic activities. Uetz et al. (2000) used protein arrays to perform large-scale yeast two-hybrid screens in an effort to identify protein–protein interactions in *S. cerevisiae*. They screened for interactions between 6000 yeast-expressed ORFs and 192 yeast proteins and were able to identify 281 putative interactions (Uetz et al. 2000). Recently, Zhu et al. (2001) used proteome chips to analyze protein activities in yeast. They made microarray slides with proteins produced by the overexpression of 5800 ORF from yeast. The arrays were subsequently used to detect protein–protein and protein–lipid interactions, which led to the identification of several new calmodulin- and phospholipid-binding proteins (Zhu et al. 2001).

5.2 Metabolomics

A full functional genomics study would ultimately need, in conjunction with gene expression data from the characterization of transcription (transcriptome analysis) or from the profiling of the proteins formed (proteome analysis), data relating to the metabolites produced (metabolome analysis) in the system of interest. Metabolomics aims at the quantification and identification of metabolites present in the sample of interest as well as the analysis of intracellular metabolic fluxes resulting from the activity of different enzymes (Fiehn 2001). The need for metabolomic studies is becoming exceedingly urgent to complement other functional studies where, despite all the achieved advances, more is yet to be

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learned. For instance, in spite of the fact that the genome of *Arabidopsis thaliana* has been fully sequenced, 30% of its genes still have not been assigned a function (Fiehn 2002). Metabolomics studies would certainly shed light on the functions of some of these genes. These studies are also highly desirable in organisms where extensive genetic characterizations have been done. The availability of annotated mutant collections renders comparative studies using metabolomic analysis very informative. This is especially true for the characterization of silent mutations where no phenotype is readily discernable (Raamsdonk et al. 2001).

Several analytic techniques are being used to perform metabolic profiling, including nuclear magnetic resonance spectrometry, mass spectrometry, and chromatographic analysis (Phelps et al. 2002). Nuclear magnetic resonance spectrometry has been popular, especially because of its nondestructive nature. In fact, it can be used to produce, *in vivo*, several spectra from the same sample at different intervals, which renders time-course studies relatively easier to complete (Ratcliffe and Shachar-Hill 2001). Nuclear magnetic resonance spectrometry has been used in several metabolic studies. Boersma et al. (2001) used nuclear magnetic resonance spectrometry to study the degradation pathway of fluorophenolics and hydroxybenzenes in microbes (Boersma et al. 2001). Raamsdonk et al. (2001) used nuclear magnetic resonance to do functional analysis using comparative genomics in yeast and were able to characterize the site of action of a silent gene in *S. cereviseae* (Raamsdonk et al. 2001). Wolfender et al. (1998) reported the *de nuovo* identification of secondary plant metabolites by coupling gas chromatography (GC) to nuclear magnetic resonance and mass spectrometry for structure analysis. For more information regarding the different applications of nuclear magnetic resonance spectrometry in metabolomic profiling in plants, please refer to the article by Ratcliffe and Shachar-Hill (2001). Mass spectrometry, though destructive, presents the advantages of faster analysis, potential for automation and high sensitivity, especially when GC/TOFMS (time of flight mass spectrometry) is used. Roessner et al. (2001) were able to quantify 100 compounds in a single run of GC/MS in a metabolomic profiling study on *A. thaliana* transgenics.

6 CONCLUSIONS

In the past few years, we witnessed tremendous progress in biology stemming from technical advances in engineering and computational sciences. The newly adopted technologies allowed biologists to devise new strategies to tackle novel questions ranging from deciphering whole genomes to dissecting complex metabolic pathways. New technologies are being introduced to render techniques used in fields such as genomics, proteomics, and metabolomics cheaper and more reproducible thus making them accessible to more researchers. The time is ripe to expend the usage of these approaches in studying filamentous fungi. Information obtained from such studies will have a huge impact on a myriad of fields ranging from medicine to agriculture to the industries where fungi are used extensively.

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6

Stability and Instability of Fungal Genomes

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1 INTRODUCTION

Examples of fungal variability abound: many phytopathological isolates from the wild display marked phenotypic and genotypic variability (Kistler and Miao 1992). Hansen (1938) described what he called "the dual phenomenon" in which conidial fungi segregate aconidial sectors; industrial yeasts are highly polymorphic for both chromosome number and length, as well as for copy number of genes encoding industrially relevant enzymes. The black *Aspergillus* species, *A. ellipticus*, persistently throws off variants initially regarded as a different species (Al Musallam 1980 and personal communication). Some fungi, e.g., *Candida albicans* (see Chapter 37 on "Candidiasis") and *Penicillium marneffei* (Borneman et al. 2000), are dimorphic, i.e., they can switch between yeast-like and hyphal forms. Dimorphism is of particular interest since it is associated with pathogenicity, nevertheless, the mechanism behind the switch is still little understood, although probably governed by changes in gene expression rather than genome instability. *Candida* also displays colony morphotype switching, in some instances associated with chromosomal polymorphism (Gow 1995).

Much of this variation is almost certainly epigenetic, but we know that the genome is prone to a variety of accidents, and is also inhabited by parasitic transposable elements, which by their very nature, cause genome instability. Fungi, like most microbes, are very directly exposed to their environment, and offer little protection to their germ lines, with the possible exception of ascomycetes whose fruiting bodies are relatively impervious to external solutes. In an extreme case, electrophoretic characterization of fungal genomes clearly shows changes in chromosome size occurring in vegetative lineages over a few generations (Davière et al. 2001).

Mutation or genome variation, is of course the starting point for evolution, and can therefore be considered essential to life, but reproducibility of an evolved life form is also the essence of life: a balance must be struck. The same is true for biotechnology, new variants may be desirable, but stability of valuable strains is also important. It is notable, however, that so long as a desirable culture can be maintained as a stock, a considerable amount of instability, e.g., of transformants, can be tolerated at the production stage (Dunn-Coleman et al. 1992). The variability of wild and industrial isolates contrasts with the relative constancy of genetic model fungi that have been selected for their uniformity of behavior and therefore provide incomplete models in this respect. Nevertheless, the ability to observe large numbers of microfungi in the laboratory, combined with much greater developmental complexity than bacteria, makes them ideal subjects for observation of natural or induced variation.

The relevance of sexual reproduction to phenotypic variability is paradoxical. While sexual recombination is expected to generate diversity, it is often the case in both plants and fungi that asexual species are the most variable. Kistler and Miao (1992) make the point that highly selected industrial strains often have a chromosomal constitution that makes them incompatible with other strains, with the result that they are sexually sterile. However, many such strains have also lost the ability to produce spores of any kind, i.e., unnecessary features have been sacrificed for industrially important traits. Thus sterility often accompanies selection for extremes. The variability of asexual species is another matter; sexual reproduction is expected to provide an almost continuous range of phenotypes from which the fittest will be selected by intraspecific competition. On the other hand an asexual population varies only by occasional mutation and mitotic chromosomal rearrangement, resulting in a discontinuous set

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of phenotypic combinations available for competition, with the result that quite divergent phenotypes may persist. Moreover, sexual reproduction can reassort beneficial genes of all sorts, whereas selection for a metabolic character gene, from the limited range available in an asexual species, may result in hitch-hiking of morphological or genomic oddities that would otherwise have been selected against.

Many issues relating to genomic stability are considered in other chapters (see especially Chapter [4] "Fungal Nuclei and Chromosomes" by B Lu; Chapter [12] "Fungal Evolution Meets Fungal Genomics" by L Leigh, E Seif, N Rodriguez, Y Jacob and F Lang; and Chapter [7] "Secondary Metabolic Gene Clusters in Filamentous Fungi" by JW Cary; Chapter [11]: "Fungal Mitochondria: Genomes, Genetic Elements, and Gene Expression" by JC Kennell and SM Cohen). Therefore, in this chapter only a few topics relating to DNA damage response and the influence of transposable elements will be discussed.

2 MUTATION AND DNA DAMAGE RESPONSE

Microbes are well suited to studies of mutation, and fungi are the obvious choice of model eukaryotic microbes, chosen on the assumption that mutation processes in these organisms will not differ greatly from those of other eukaryotes. Research shows that while some fungi possess special mechanisms such as repeat induced point mutation (RIP, see below), and DNA repair systems may differ in detail, a number of homologous mechanisms for dealing with damaged DNA are detectable in all organisms studied.

2.1 Mutant Induction

Mutations can readily be induced using standard mutagens. Ultraviolet light is easy to use, but has the disadvantage that, like gamma and X-radiation, it induces chromosomal rearrangements, which may hamper subsequent genetic investigation (Kafer 1965). Nitroquinoline oxide (Bal et al. 1977) is recommended as an effective but readily inactivated mutagen. There are advantages also in using minimal mutagen doses (Bos 1987) or relying on spontaneous mutation (Teow and Upshall 1983). It should be noted that while some mutagens, e.g., nitrous acid, directly convert bases to forms that mimic other bases on replication, many other mutagens result in lesions that block replication and only become heritable changes as a result of inaccurate repair.

2.2 DNA Repair

This is a complex area of research, which has progressed further in yeasts and mammals than in filamentous fungi, although important information has come from mutants in *Aspergillus nidulans* and *Neurospora crassa*. Here only the processes believed to be involved are summarized and the reader is referred to reviews by Elledge (1996); Wood (1996), and Goldman et al. (2002) for more complete accounts.

2.2.1 Repair Pathways

Initial information on DNA damage responses of fungi came from characterization of radiation or mutagen-sensitive mutants, e.g., the *rad* series in *Saccharomyces cerevisiae*. In addition, some classes of mutants with cell-cycle abnormalities turn out to be hypersensitive to mutagens as a result of failure of checkpoints which should have given the cell time for DNA repair. Genome analysis in yeasts and other fungi, as well as higher eukaryotes, has expanded the list of genes involved. Mutagen-sensitive mutants in a variety of fungi have been classified into epistasis groups by examination of double mutants, the principle of the method being that pairs of mutants with defects in the same pathway will show only the phenotype of the single components, but pairs of mutants in different pathways will act additively to give a phenotype more defective than either component alone (e.g., Kafer and May 1998). This procedure is not guaranteed to detect all repair pathways, particularly in cases of genetic redundancy, and may give uninterpretable results where mutation results in more complex phenotypes than loss of function. In addition, mutations in regulatory systems may interact with more than one repair pathway. The alternative approach to analysis of DNA repair is by genome analysis, relying on sequence similarities to genes of known function, and therefore dependent on previous biochemistry and classical genetics for identification of the reference sequences. Methods for detecting protein interactions can then be used to identify new functional partners of known proteins. The most detailed analysis of DNA repair is the bacterial SOS system, but it is apparent that, while homologies to bacterial proteins are often found in eukaryotes, they do not necessarily reflect identical functions.

Bacterial experience has led to the expectation of a variety of DNA repair systems in fungi. The simplest form of repair merely reverses the damage process. Fungi are likely to be exposed to UV irradiation as a component of sunlight, and to counter this in the same environment, eukaryotes have acquired light-dependent photolyases that split pyrimidine dimers, which are the primary products of UV irradiation, thus directly reversing the damage. Photolyase genes have been identified in *N. crassa*, *Trichoderma harzanium*, and *S. cerevisiae*. Similarly methyltransferases can remove methyl groups from DNA bases, reversing the effects of DNA alkylating agents. Methyltransferases have been assayed in *A. nidulans* (Swirsky et al. 1988) and methyltransferase-encoding genes have been identified in yeast (Wood 1996).

The next simplest repair processes starts by recognition and detachment of defective bases from the sugar-phosphate backbone by glycosylases specific for each type of defect

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(reviewed in McCullough et al. 1999). The resulting apurinic/apyrimidinic (AP) site is then processed by AP-specific endonucleases and lyases to generate a gap; other repair systems (nucleotide excision repair, long-patch repair and mismatch repair) remove stretches of DNA differing in length according to the system. In all these cases the excised region is replaced using a replicative DNA polymerases to copy the undamaged partner, and the ends are rejoined by a ligase. The number of overlapping systems of this type has made genetic analysis difficult, e.g., mutants identifying two parallel systems of excision repair have been found in *N. crassa*, but no such mutants have been isolated from *A. nidulans*, although homologous of the relevant genes can be identified in the genome (Goldman et al. 2002).

If both DNA strands are damaged, e.g., by double-strand breaks such as are induced by ionizing radiation, the lesion is more difficult to deal with, and can only be repaired accurately by mechanisms employing recombination, or more precisely, gene conversion (reviewed by Haber 2000). In cells in the G2 phase of the cell cycle, a sister chromatid can be used as template, or diploid cells can use the second homologous chromosomes. It has also recently become apparent that recombinational repair is a regular and necessary accompaniment to DNA replication in bacteria (Cox 2001): in short, whenever a replication fork pauses at a blockage, replication skips the damaged stretch on one strand, but is allowed to continue on the other. A recombinational mechanism then repairs the gap in the first daughter duplex using the second as template.

Finally, in the absence of homologues usable in this way, or where the system is overloaded, the best that can be done is to repair the defect without regard to the correct sequence, i.e., by error-prone mechanisms. Double-strand breaks can be repaired by nonhomologous end-joining (NHEJ), which creates deletions, translocations, or inversions, and recent research has uncovered a series of sloppy DNA polymerases that can fill gaps irrespective of damage to the template (Goodman 2002).

2.2.2 Damage Detection, Signal Transduction, and Cell Cycle Checkpoints

The complex phenotypes of some mutants imply that loss of one repair pathway puts extra burdens on others, e.g., *A. nidulans uvsF* and *uvsH* mutants, characterized as defective in error-free postreplication repair (Goldman et al. 2002), also exhibit increased mitotic recombination and reduced meiotic fertility (Kafer and May 1998). In other instances, mutant phenotypes are ambiguous, which could mean that the gene responsible has roles in more than one pathway, or in damage detection or transduction of the resulting signal to repair pathways and cell cycle checkpoints. Glycosylases are able to detect the specific types of damage to which they respond (McCullough et al. 1999). A more general mechanism of DNA damage detection employs a DNA sliding clamp protein, Rad1, which processes along the DNA and is similar to the PCNA replication protein (Thelen et al. 1999). The role of the damage detection system is to prime a classical protein kinase transduction signal chain to activate multiple repair pathways, and at the same time to put in place inhibitory checkpoints that stall the cell cycle until repair is completed.

In mammals and fungi a normal checkpoint inhibits the start of mitosis until replication is complete. In the presence of DNA damage, the best understood effect on the cell cycle is prolongation of this delay. The mechanism appears to depend on inactivation of similar proteins by phosphorylation in *Schizosaccharomyces pombe*, *A. nidulans*, and mammals, but employs different proteins in *S. cerevisiae* (Goldman et al. 2002). A fruitful method of investigation of the components of this and other cell cycle controls in *A. nidulans* has been the isolation of suppressors, which reverse the effects of other mutants, by altering the interactions of the encoded proteins (e.g., McGuire et al. 2000; Kraus and Harris 2001). Mutants isolated in this way are affected in mitotic timing, but also in septation, the equivalent of cell division in higher eukaryotes.

2.2.3 Stress-Induced and Adaptive Mutation

There is evidence of some cross-talk between DNA repair and other stress-induced pathways in *S. pombe.* (Pearce and Humphrey 2001). There is also plenty of evidence from bacteria that mutation rates increase during starvation-induced stationary phase; what is more debatable is whether such mutation is general or adaptive, i.e., directed towards relief of starvation (Foster 2000). More recently, Yeiser et al. (2002) have demonstrated a clear advantage, under competitive conditions, for *Escherichia coli* strains that are wild-type for three stress-induced, error-prone DNA polymerases. Their superiority depends on both the continuation of DNA replication under stress and the production of mutations conferring growth advantages in stationary phase ("GASP").

Similar phenomena have been observed in less detail in *S. cerevisiae* (e.g., Marini et al. 1999), while in *C. albicans* starvation induced by provision of L-sorbose as sole carbon source results in increased mitotic nondisjunction. In the case investigated (Janbon et al. 1999) it appears that the result is adaptive because nondisjunction products include monosomics for chromosome V in which a control mechanism is released to allow utilization of sorbose. Kafer (1987) also found that all amino acid deficient mutants of *A. nidulans* were hypersensitive to certain mutagens, suggesting that cross-pathway control of amino acid supply may affect DNA repair, and confer the potential advantage of increased mutation under conditions of stress.

Many biotechnological applications of fungi place the organism under unusual conditions that come under the general heading of stress. It is therefore important to expect adaptive mutations to accumulate under these conditions, and to recognize that the resulting changes may be beneficial, e.g., in allowing better growth, or harmful, if they deflect metabolism away from the desired product.

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2.3 Chromosome Rearrangements

Chromosomes can become rearranged by a number of mechanisms, the most straightforward of which is mutational breakage and reunion. Recombinational mechanisms are also important, and it is evident that the cell must be able to guard against recombination between the many repeated sequences providing local homology in nonhomologous locations. Forces for the maintenance of the existing gene order are harder to envisage, but must be considered for gene clusters (see Figure 1 and Chapter 7). More remarkable is the observation of microsynteny between chromosomes of *Magnaporthe grisea* and *N. crassa*, organisms probably separated by 200 million years (Hamer et al. 2001). In this case also, synteny within a 53 kb region does not imply total conservation of gene order (see "Transposon-Induced Damage").

Unrepaired double-strand breaks that cannot be repaired by recombination with a homologue may be joined together randomly ("non-homologous end joining"), to create translocations or inversions. In *Drosophila* and in fungi there are also many known instances of apparent telomeric attachment of broken ends, to create terminal translocations (Novitski et al. 1981; Burr et al. 1982, although it is a dogma that only broken ends can rejoin. For this reason, such translocations are sometimes referred to as "quasi-terminal" (Perkins 1997), see "Telomeres." When strains carrying chromosomal aberrations cross with normal strains, the progeny, particularly in the case of insertional or terminal translocations, includes those with deletions and others with duplications. Deletion–duplication strains can arise in a single step by the break-induced replication pathway for repair of double-strand breaks, in which a chromosomal segment distal to a break is replaced by replication of a nonhomologous template (Haber 2000).

Large deletions are usually lethal (but see Caddick et al. 1986 for a viable 340 kb telomeric deletion), while duplications are often tolerated, albeit with some loss of fitness. Duplication strains of *N. crassa* are sexually barren (Perkins 1997), a condition originally ascribed to RIP (see later), but now explained by MSUD (Meiotic Silencing by Unpaired DNA), see Shiu et al. 2001. Vegetative colonies of duplication strains commonly throw off faster-growing sectors from which one or other of the duplicated segments has been lost. Particularly in the case of terminal translocations, the telomeric junction appears to be relatively unstable so that the abnormally attached copy is lost more frequently than the normal one (Perkins 1997). Loss of duplicated segments in *A. nidulans* has been studied in some detail (Birkett and Roper 1977), as has the occurrence of other, physiologically unexplained sectors with deteriorated morphology, that frequently arise in *A. nidulans* duplication strains (Azevedo and Roper 1970). Extensive studies of chromosomal aberrations by means of ascus analysis in *N. crassa* have been made possible by the fact that deletion progeny in crosses heterozygous for aberrations are conspicuous as colourless nonmaturing ascospores (Perkins 1997). In *A. nidulans*, similar crosses can be recognized by the high proportion of aneuploid progeny (Kafer 1977).

2.3.1 Chromosome Disruption by Recombination Between Repeated Sequences

Recombination between repeated sequences can generate either duplication or deletion of the intervening DNA (Fierro and Martín 1999). Prime examples of the importance of such events for biotechnology is the duplication or deletion of the penicillin biosynthesis cluster in *Penicillium chrysogenum* (Fierro et al. 1995; 1996) and variation in copy number of carbohydrate utilization genes at yeast telomeres (Codón et al. 1998). Petes and Hill (1988) comprehensively reviewed recombination between repeated genes. Homologous integration of a plasmid containing a chromosomal sequence generates a tandem repeat on either side of the integrated vector. This simple source of tandem repeats has been used in a number of experimental systems. In *S. cerevisiae* tandem repeats undergo three basic types of recombination: gene conversion, altering the sequence of one of the repeats to conform with the other, "pop-out", in which reciprocal recombination between the repeats results in excision of one repeat and the intervening sequence (usually vector, in the case of transformation integrants), and finally unequal crossing over, resulting in the formation chromosomes containing single and triple copies as a result of mispaired recombination between the two-copy chromosomes. In yeast gene conversion is usually much more frequent than crossing-over, and all these events occur approximately 1000 times more frequently during meiosis than during mitosis. Mitotic gene conversion in *A. nidulans* of tandem duplications of the *benA* gene was 2.6×10^{-4}, while mitotic pop-out occurred at a similar frequency: 2.0×10^{-4} (Dunne and Oakley 1988). Meiotic frequencies are higher, but similarly proportioned: transformation-induced tandem duplications of the *brlA* locus undergo both gene conversion and pop-out at frequencies between 9 and 14% (Clutterbuck and Stark, unpublished). Recombination between dispersed repeats, e.g., transposons, are likely to have more a drastic effect on chromosome constitution (Petes and Hill 1988). In *S. cerevisiae* recombination between mutant copies of the *ARG4* gene depended on location (Goldman and Lichten 1996), ectopic meiotic recombination was much less frequent than homologous recombination, and was lower still when the repeats were on different chromosomes. One interesting finding was that repeats close to nonhomologous telomeres recombined considerably more frequently than those elsewhere. The ability of the cell to distinguish different contexts for repeated sequences implies a homology searching mechanism that surveys relatively long DNA tracts. More detailed scrutiny of the degree of homology is implied by the finding that the mismatch repair system in yeast, as in *E. coli*, has an additional role in determining the extent of similarity of potential homologues (Chen and Jinks-Robertson 1999), such that mutations in this pathway allow recombination between sequences that would otherwise be rejected.

2.4 Chromosome Number

The majority of the true fungi are haploid or dikaryotic; indeed some authors have included this feature in the definition of the fungal kingdom (see Leigh et al., this volume). Nevertheless, many yeasts, including *S. cerevisiae* and *C. albicans*, are found in nature as vegetative diploids or polyploids, and other fungi, e.g., *Basidiobolus ranarum* (Robinow 1963) have such oversized nuclei that polyploidy is more than likely. Yeast diploids are very stable, but segregate occasional variants as a result of mitotic crossing over. In other fungi, e.g., *A. nidulans*, diploids may rarely be found in the wild (Upshall 1981) and are only moderately stable in the laboratory, undergoing mitotic recombination and breaking down by chromosomal nondisjunction, via aneuploidy, to stable haploids (Kafer 1961). Diploids have been isolated from many other fungi (Caten and Day 1977), and been used in genetic analysis via the parasexual cycle, but have never been found in *N. crassa* (Davis 2000).

Aneuploid nuclei can readily arise by spontaneous chromosomal nondisjunction, and are a source of unstable defective phenotypes. In *A. nidulans* a number of high frequency of aneuploidy (*hfa*) loci have been identified by temperature-sensitive mutants that frequently throw off morphologically distinct aneuploid sectors (Hughes et al. 2000). Similar phenotypes are also associated with some mutants originally identified as having defects in mitosis that result in accumulation of nuclei with increased ploidy (De Souza et al. 1999). Finally, meiotic nondisjunction is frequent in crosses between strains differing with respect to chromosomal translocations, giving rise to aneuploids for the chromosomes concerned, an observation which has been used as a diagnostic feature of translocated strains (Kafer 1977). Aneuploids present similar phenotypic defects to those of other duplication strains discussed above. In *A. nidulans* all eight disomic phenotypes can be distinguished, as can trisomics arising in a diploid background (Kafer and Upshall 1973). Loss of the extra chromosome by a second nondisjunction event has selective advantage and is relatively frequent, however disomics can be stabilized by certain mutations, the best characterized of which is *sod*^{VI}*C1*. This turns out to encode a partially defective copy of a gene essential for growth, whose expression is increased by disomy of chromosome IV on which it is situated (Whittaker et al. 1999). Compare this with the case of monosomy in *C. albicans* allowing sorbose utilization, as discussed earlier in "Stress-Induced and Adaptive Mutation").

Disomic aneuploids in *N. crassa* are extremely unstable; they can be formed by nondisjunctive meiosis, but are only detected by the presence of "pseudowild" progeny of crosses between strains carrying closely linked auxotrophic markers. These can complement in the disomic but will rarely give wild type progeny by recombination. The pseudowild progeny are in fact heterokaryons of the two haploid breakdown products of the disomic, each carrying one of the auxotrophic markers, which again complement to give a prototrophic heterokaryon (Pittenger 1954). In contrast to this, baker's and brewer's yeasts are highly polymorphic for chromosome number, ploidy typically varying between 1.3n and 3n (Codón et al. 1998). It seems probably that duplication of whole chromosomes has been selected during industrial adaptation, possibly facilitated by stress-induced mitotic accidents (see earlier).

3 TELOMERES

Due of the inability of the normal replication process to fill in DNA ends, telomeres are potentially liable to progressive shortening; moreover, if not protected, they are free to undergo end-joining with other telomeres or broken chromosomes. However, a well-regulated complex of processes (reviewed by McEachern et al. 2000) normally ensures protection of the ends and stabilization of telomere length. Many fungi have the tandem repeat sequence (TTAGGG) that is also found in vertebrates, but yeasts have slightly different and more variable repeats. A complex of cap proteins binds to the repeats and prevents attack by exonucleases or components of the NHEJ pathway. The most important constituent of the cap is telomerase, a ribonucleoprotein that adds fresh repeats during S-phase, using a short stretch of the RNA component as template. Telomere length is apparently measured in *S. cerevisiae* by the binding of a specific multifunctional protein, Rap1.

Excessive telomere shortening leads to cell cycle arrest and cessation of growth. However, an additional process of telomere maintenance is experimentally detectable when telomerase is defective. This mechanism uses homologous recombination to restore multiple telomeric repeats, and in some cases subtelomeric sequences also, suggesting that uncapped telomeres behave like double-strand breaks in inducing recombination. McEachern and Iyer (2001) found that in a telomerase-defective strain of *Kluyveromyces lactis*, the telomeric regions become highly recombinogenic. The result of this was that telomere restriction patterns diminished in variety during vegetative growth, implying that some telomeres had acquired the patterns of other telomeres by gene conversion. In addition, a *ura3* gene inserted just upstream of the telomeric repeats was lost from some subcultures, and in other subcultures spread to the telomeres of up to a third of the remaining chromosomes. Unequal crossing over between mispaired telomeres may also occur, leading to shortening of one telomere accompanied by elongation of another.

Finally, if all telomere protection systems fail, a telomere will continue to behave as a double-strand break and become susceptible to NHEJ, resulting in translocation or chromosome fusion. All of these abnormal processes have been detected in mutants defective in the standard telomerase maintenance system, but it is not difficult to imagine that they could be causes of chromosome instability under stress.

A more remarkable property of telomeres is their function as a reservoir of double-strand-break-repair proteins

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(reviewed by Cooper 2000). These proteins, whose likely function is to protect free DNA ends, are released from telomeres when double-strand breaks are detected in the cell. They include Ku proteins that can bridge double-strand gaps, and Sir proteins that are well known as telomere-associated transcriptional silencers, and in this context are postulated to prevent disturbance of the repair process by RNA polymerase. In addition to the tandem short telomeric repeats, most telomeres have a subtelomeric region harboring additional heterogeneous repeats, including many transposable elements, and in some cases active genes. Codón et al. (1998) found that bread and wine yeasts varied widely in the number of copies of carbohydrate metabolism genes (e.g., *suc* and *mal*), in proportion to encoded enzyme activities. They suggest that this variability relates to the ability of transposable elements, clustered in these regions, to produce chromosome rearrangements (see later).

4 TRANSPOSABLE ELEMENTS

Forty-five percent of the human genome consists of identifiable transposable elements and the remainder probably includes many unrecognizable transposon remains (Ostertag and Kazazian 2001), The comparable figure for *S. cerevisiae* is 3.1% (Dujon 1996); the slightly larger genomes of most filamentous fungi may include more transposons, but it seems likely that restrictions on nuclear size may provide a selective force for limitation of repetitive DNA in fungi. Nuclear size is closely related to cell size (Gregory 2001), and small spores are likely to benefit aerial dispersal; in addition, small nuclei should be advantageous for nuclear migration in filamentous cells.

Most of the types of transposable element found in higher eukaryotes are also present in fungi (Daboussi 1996; Kempken and Kück 1998). The majority of transposons are usually found to be mutated and inactive, but active elements have been found in *Ascobolus immersus* (Goyon et al. 1996; Kempken 2001), *Botrytis cinerea* (Levis et al. 1997), *Fusarium oxysporium* (Hua-Van et al. 2000), *Magnaporthe grisea* (Nakayashiki et al. 1999a), *Mycosphaerella graminicola* (Goodwin et al. 2001), and *Neurospora crassa* (Kinsey et al. 1994), where in many cases they are associated with phenotypic instability. In *M. graminicola*, as in many other cases, the element was detected by a probe used for strain fingerprinting. Comparison of asexual and sexual progeny indicated that the element could transpose in both stages of the life cycle, but was more active during the latter. This contrasts with the conclusion for the human genome that despite their frequency, transposable elements have not been active for 50 Myr (International Human Genome Sequencing Consortium 2001).

4.1 Transposon-Induced Damage

Transposons can disrupt the genome in a number of ways (Ostertag and Kazazian 2001): they may insert into important coding or regulatory sequences, or may impose additional regulatory features on nearby genes; homologous recombination between transposon copies at different sites can rearrange chromosomes ("Chromosome Disruption by Recombination Between Repeated Sequences"); composite transposons may be formed from nearby single elements, resulting in the movement of the intervening DNA as part of the new transposon; finally, some transposable elements can cause local rearrangement of neighbouring DNA during transposition.

Davière et al. (2001) found that transposable elements were particularly active in one phenotypically unstable strain of *Fusarium oxysporium*, and that clonally related strains frequently differed in length of some chromosomes, and in one case their presence or absence. Eight out of twelve chromosomes showed such polymorphisms, and instability of each chromosome correlated with transposon density; however, transposition in or out of a target *niaD* gene was not itself accompanied by rearrangement. The authors therefore concluded that rearrangements are probably the result of ectopic vegetative recombination between repeated elements (see "Chromosome Disruption by Recombination Between Repeated Sequences").

It has long been a puzzle that while evolutionary influences in favor of clustering of genes of related function are debatable, and those that are postulated do not appear to be very powerful (this volume, Chapter [7] JW Cary: "Secondary Metabolic Gene cluster in Filamentous Fungi"), the susceptibility of clusters to scattering by chromosome breakage and nonhomologous rejoining is very clear. More remarkable still is the evidence (Figure 1 and Chapter 7 (JW Cary) Figure 2) that the gene order within clusters can become reshuffled without dispersing the components. Seoighe et al. (2000) compared the genomes of two yeasts, *C. albicans* and *S. cerevisiae*, which are believed to have separated 140–330 million years ago, concluding that local inversions had occurred approximately as frequently as larger rearrangements. Large-scale rearrangements are assumed to be the result of random chromosome breakage and reunion, but the mechanism of local rearrangement is less obvious. The aberrant behavior of some transposable elements seems likely to provide the best answer.

A clear explanation of a way in which local inversions and deletions could be caused by aberrant excision and reintegration of an *impala* (*Tc1-mariner* family) element are described for *F. oxysporium* by Hua-Van et al. (2001). Excision of nonreplicating class II transposons requires interaction between the two ends of the elements, resulting in chromosome breakage at those ends (Figure 2, panel 1). If two such elements have inserted close together on a chromosome, a pair of ends from different elements may interact, resulting in a chromosome break flanked by the two elements. These retain the ability to interact with each other and re-insert jointly into fresh DNA, but they remain tethered to their parent chromosome (Figure 2, panel 2), therefore re-insertion is likely to be close to the original site (Figure 2, panels 3–4). Repair of the nontransposon chromosomal ends from

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the initial excision process results in one inversion (Figure 2, panel 2), while re-insertion may cause either a second inversion (Figure 2, panel 3) or deletion (Figure 2, panel 4), depending on the relative orientation of the two transposon ends. Similar mechanisms can be expected to operate with any type II (DNA-mediated), nonreplicative element that transposes by excision.

Excision may also occur by homologous recombination between repeated terminal components of transposons. Depending on the type of element, excision by either mechanism can leave a footprint derived from a target site duplication or terminal repeats. These may, or may not, interfere with gene expression, but are often sufficiently innocuous for excision events to be detected as reversion of a mutation originally caused by transposon insertion.

4.2 Fungal Defense Against Transposons

Two types of defence against multiplication of repetitive DNA are found in fungi, reviewed by Selker (1997); Cogino (2001). Both probably depend on recognition of homology of the copies, but while the first alters the DNA temporarily to prevent transcription, or permanently, rendering the transcripts meaningless, the second acts post-transcriptionally to destabilize the mRNA.

4.2.1 RIP and MIP

Neurospora crassa possesses an effective mechanism for mutating repeated genomic elements: RIP produces multiple G:C–A:T transition mutations in repeated sequences during a specific premeiotic stage of sexual reproduction (Selker 1990). Both copies are mutated, the process applies with high efficiency to all repeats longer than 400 bp, and acts most strongly on tandem copies. The mechanism of RIP is still uncertain, but is postulated to involve deamination of methyl cytosine to thymidine (but see later). G-A transitions are therefore assumed to represent C-T changes on the opposite strand. For any one repeat, G-A and C-T transitions may differ significantly in frequency, implying that each strand is mutated independently. Furthermore the mutated region may extend beyond the repeat, suggesting that the mutation generator processes along each strand, and may overshoot the repeat region.

While nearly all isolates of *Neurospora* species possess many degenerate copies of the *Tad* LINE-like retrotransposon, only one isolate, the Adiopodoumé strain, possesses active versions (Kinsey et al. 1994). Anderson et al. (2001) found that these elements were rapidly inactivated by RIP during sexual reproduction, implying that RIP is an effective mechanism for immobilizing transposable elements, including in the Adiopodoumé strain. One possible explanation for the survival of active elements in this isolate is that it had not been through a sexual stage since it received its *Tad* element from an unknown source. A similar, but much rarer process, occurs in *Podospora* (Graïa et al. 2001). In *N. crassa* it has been observed that late-maturing ascospores are most likely to show evidence of RIP (Singer et al. 1995), and the single *Podospora* colony in which evidence of RIP was detected, came from an spore ejected from a late-maturing ascus. This could be because these nuclei have spent longer in the dikaryon phase when RIP occurs (Selker 1990).

RIP in *N. crassa* is paralleled by MIP in *Ascobolus immersus* (Goyon and Faugeron 1989). This process also affects duplicated sequences and results in gene inactivation by DNA methylation during the dikaryon stage, however, it does not result in mutation, and methylation is reversible. The relationship between RIP and cytosine methylation in *N. crassa* is complex. *N. crassa* sequences with more than 1% RIP mutations become methylated during subsequent vegetative growth, but it has not been possible to detect DNA methylation at the dikaryon stage, and mutation of the *dim2* gene, encoding methyltransferase, abolishes vegetative DNA methylation without affecting RIP (Kouzminova and Selker 2001).

Transition-mutated copies of transposable elements have been seen in a number of other fungi in which the RIP mechanism has not otherwise been demonstrated (despite many opportunities in the case of *A. nidulans*): *Aspergillus*

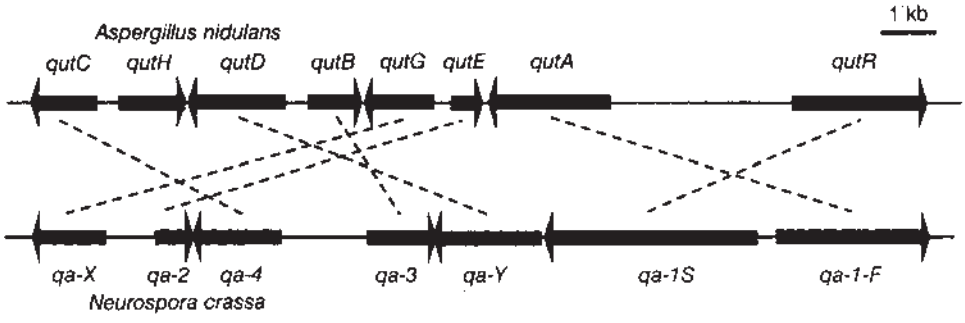

Figure 1 The genes for quinate degradation in *A. nidulans* and *N. crassa* have remained as a cluster despite shuffling of gene order and orientation. Arrows represent transcribed genes; dashed lines join genes of like function. No orthologue of *A. nidulans qutH* has been found in *N. crassa*. Reprinted, with permission, from Fig. 12.2, Clutterbuck (1995).

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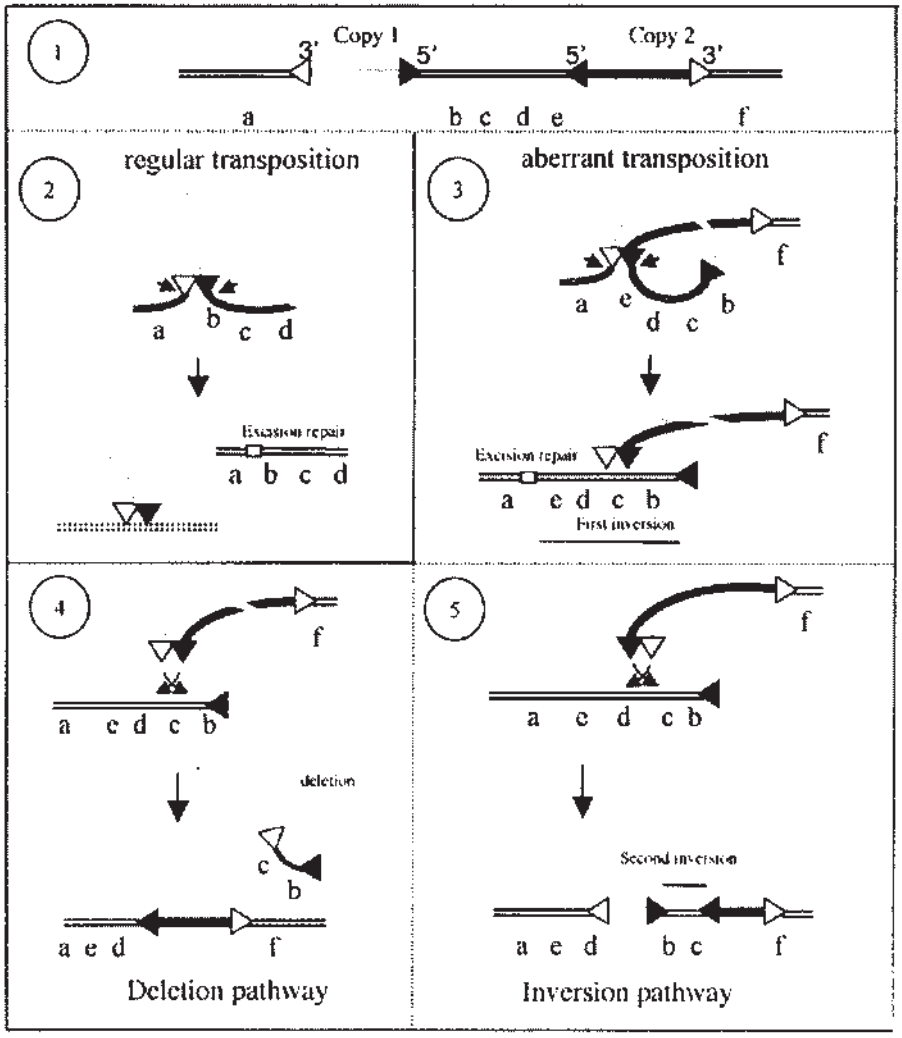

Figure 2 Model for generation of inversions and deletions by aberrant transposition. Flanking genomic subregions are labeled a–f. The model requires two linked elements, copy 1 and copy 2 in inverted orientation (panel 1). Normal transposition involves synaptic complex formation between transposase and inverted terminal repeats (ITRs) from the same element (panel 2). Endonucleolytic cleavage occurs at the junction between ITR and genomic flanking sequences. The synaptic complex is then free and can reinsert elsewhere in the genome. Aberrant transposition is achieved by synaptic complex formation between the 3′ ITR (open arrowhead) of copy 1 and the 5′ITR (filled arrowhead) of copy 2 (panel 3). In both cases endonucleolytic cleavage leaves a gap which is filled by cellular repair mechanisms (open rectangle), but in the aberrant version a segment of genome has been inverted, and the two transposable elements remain tethered to the broken chromosome ends, which are therefore obliged to reinsert close by (panels 3–5). In one orientation of the two ends, reinsertion gives rise to a deletion (panel 4), while in the other it creates a second inversion (panel 5). Reprinted, with permission, from Fig. 6, Hua-Van et al. (2002).

fumigatus (Neuveglise et al. 1996), *A. nidulans* (Aleksenko and Clutterbuck 1996; Nielsen et al. 2001), *F. oxysporium* (Hua-Van et al. 1998), *Magnaporthe grisea* (Nakayashiki et al. 1999b) and *Mycosphaerella graminicola* (Goodwin and Tian 2002). Laboratory strains of *A. nidulans* contain five copies of the Mobile *Aspergillus* Transformation Enhancer (MATE) element (Aleksenko and Clutterbuck 1996, Clutterbuck unpublished). Three of these are almost identical, while two show numerous GC to AT transitions relative to these two, strongly suggesting a RIP mechanism. Variation in susceptibility to RIP may be determined by chromosomal location (Aleksenko et al. 2001). This is paralleled by the *Hideaway* element of *Ascobolus immersus* (Kempken 2001) that avoids MIP methylation by its location in the rDNA repeat region which, although itself highly repetitive, is immune to RIP (Haack and Selker, quoted in Selker 1997).

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Methylcytosine could not be detected in *A. nidulans* (Antequera et al. 1984) or *A. fumigatus* (Neuveglise et al. 1996), but it has recently been found at very low levels in *Aspergillus oryzae* (Gowher et al. 2001). It therefore remains possible that RIP results from specialized cytosine methylation occurring only under particular circumstances. These circumstances might be assumed to be those prevalent only at the dikaryotic stage of sexual reproduction, but this leaves the dilemma of how to explain evidence of RIP-like mutation in asexual species such as *A. fumigatus* (Neuveglise et al. 1996) and *F. oxysporium* (Hua-Van et al. 1998). There are two obvious possibilities: either RIP can also occur at low frequency under other conditions, e.g., accompanying mitotic crossing over, or the sequences showing evidence of RIP are relics of earlier times when these species retained a sexual phase.

The premeiotic timing of RIP suggests that it may use the meiotic chromosomal pairing mechanism to identify duplications in pre-fusion haploid nuclei. Tandem repeats, such as are regularly produced by homologous integration of transforming DNAs, but may also arise by multiple transposon insertion, can also be eliminated by "pop-out" recombination between the two copies. Examination of *Podospora anserina* asci shows that loss of duplications by this mechanism also occurs at the two-strand stage, i.e., before premeiotic DNA replication in the dikaryon (Picard et al. 1987).

4.2.2 Post-Transcriptional Gene Silencing

Transposon activity can also be prevented by interference with expression of multi-copy genes. Gene silencing in plants is well known, if still incompletely understood, and has features in common with, but not identical to the phenomenon of "quelling" and "MSUD" in *N. crassa* (Cogino 2001, Shiu et al. 2001). Work on quelling has mostly concerned the *al-1* and *al-3* spore-color genes. Multiple copies of these genes, or fragments of them, created by homologous transformation, prevent expression of the resident copy, resulting in an albino phenotype. The fragments can be as small as 132 bp, and must be from the transcribed sequence. Transcription itself is not affected, but mature transcripts are much reduced, while small (25 base) sense and antisense transcripts are also seen in quelled strains (Cogoni et al. 1996). Surprisingly enough, quelling appears to be dominant in heterokaryons, evidently mediated by a cytoplasmic factor, possibly the small RNAs. More recently (Catalanotto et al. 2002) mutants deficient in quelling have identified three *qde* genes; *qde-1* and *qde-3* both lack the small RNAs; the former shows homology to RNA-dependent RNA polymerases, and the latter to helicase. The *qde-2* mutants retain the small RNAs, but are defective for subsequent breakdown of the mRNA and the *qde-2* gene shows homology to one of similar function in *Drosophila*.

It can be assumed that the function of quelling is to prevent expression of transposable element genes required for their transposition. In *S. cerevisiae* a large number of loci are involved in dampening *Ty1* transposition (Scholes et al. 2001); many of these also have other roles in genome maintenance, e.g., responding to DNA damage or controlling telomere length. A more general relationship between transposable elements and chromosome maintenance is discussed by Labrador and Corces (1997), including the widespread accumulation of these elements at telomeres, and in *Drosophila*, their substitution for telomeric repeats of other organisms (Figures 1, 2).

5 CONCLUSIONS

The genome can be seen as three-cornered battleground between ordered organismal coding, parasites in the form of transposable elements, and the forces of chaos. Biotechnologists require ordered gene function, redirected towards an industrial end, but for that purpose the chaotic power of mutation is an essential tool, if kept under control by efficient DNA repair systems, and even transposable elements can be harnessed as investigative tools. The wealth of variation in natural systems, often amplified under pressures exerted by biotechnology, is evidence of the extremes that cultured organisms can be driven to, if such evidence were needed. On the other hand, the wiles of biological systems in counteracting applied pressures are something all biotechnologists must be aware of.

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7

Secondary Metabolic Gene Clusters in Filamentous Fungi

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1 INTRODUCTION

Filamentous fungi produce a number of secondary metabolic compounds that have been shown to be both of great value (i.e., antibiotics and anti-hypercholesterolemics) and great harm (i.e., aflatoxins and trichothecenes). Since the initial discovery of clustered genes involved in the biosynthesis of penicllin in *Penicillium chrysogenum* and *Aspergillus nidulans* (Diez et al. 1990; MacCabe et al. 1990), a number of secondary metabolic gene clusters have been identified and characterized in filamentous fungi at the molecular level [reviewed in Cary et al. (2001); Keller and Hohn (1997)]. In general, fungal secondary metabolic pathway gene clusters share common genetic components with respect to function. In addition to the structural genes encoding the biosynthetic enzymes the pathway cluster can also contain a gene(s) required for regulation of expression of the other pathway genes (Chang et al. 1995b; Pedley and Walton 2001; Proctor 2000) and a gene(s) required for export of the metabolite or "self protection" to the metabolite (Alexander et al. 1999; Pitkin et al. 1996). A common question that inevitably arises during the study of fungal gene clusters has been why are the genes for these biosynthetic pathways maintained as a cluster(s) in the genome and what advantage does this impart on the fungus? This is especially perplexing in light of the fact that all of the secondary metabolic gene clusters studied appear to be "dispensible" with respect to their metabolic functions and advantage imparted to the producing organism. Keller and Hohn (1997) used the term "dispensable metabolic pathways" for those primary and secondary metabolic pathways "that either are not required for growth or are only required for growth under a limited range of conditions."

A number of hypotheses have been put forth to rationalize clustering of genes involved in secondary metabolism. One school of thought states that gene clustering may be for the purpose of coordinated gene expression through sharing of regulatory elements. Another hypothesis is based on evolutionary evidence that supports the horizontal genetic transfer of metabolic pathways from prokaryotes to fungi and subsequently from fungi to fungi. Additional support for the role of horizontal gene transfer (HGT) for the presence of gene clusters in fungi is set forth in the commentary of Walton (2000). All of these hypotheses are discussed further in Section 4. In addition to discussing possible reasons for the existence and apparent maintenance of secondary metabolic gene clusters in fungi, this review will provide key information and references on the molecular characterization of secondary metabolic pathways including (a) antibiotics, mycotoxins, etc., and (b) host-specific toxin biosynthetic pathways. All of these pathways consist of a group of genes that are clustered in one or several fungal species. In some cases the gene clusters have only been partially characterized and information may not be available on all presumed biosynthetic, regulatory, and transporter/self-protection genes (Table 1). Where these features have not been reported, they may not exist on the cluster and are not closely linked, or they have not been discovered so far.

2 SECONDARY METABOLIC PATHWAYS

2.1 Aflatoxins and Sterigmatocystin

Aflatoxins are toxic and carcinogenic polyketide-derived secondary metabolites that are produced by a number of species of *Aspergillus*. Included are numerous isolates of *A. flavus*, *A. parasiticus*, and *A. nomius*, two isolates of *A. pseudotamarii* (Ito et al. 2001), nine of *A. bombycis* (Peterson et al. 2001) as well as one isolate of

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Table 1 Secondary metabolic pathway gene clusters in filamentous fungi[a]

Pathways	Approx. cluster size (kb)	No. of ORFs[c]	Genetic composition of the cluster[b]		
			Regulatory genes[d]	Structural genes	Transport genes
1. Secondary metabolite					
Aflatoxins	75	23	1	21	1
Sterigmatocystin	60	26	1	15	0
Trichothecene	26	12	3	8	1
Penicillin	15	3	0	3	0
Cephalosporin					
Early	15	2	0	2	0
Late	5	2	0	2	0
Gibberellins	17	6	0	6	0
Melanin					
A. alternata	30	3	0	3	0
A. fumigatus	19	6	0	6	0
Fumonisins	20	5	0	5	0
Paxilline	50	17	2	9	1
2. Host-specific toxin					
HC-toxin	600	7	1	5	1
AK-Toxin	5	4	1	3	0

[a] Modified from Keller and Hohn (1997).
[b] Gene function determined or based on identity to other known genes.
[c] ORFs (open reading frames).
[d] Regulatory gene present within gene cluster.

A. ochraceoroseus (Frisvad and Samson 1999; Klich et al. 2000) and *Emericella venezuelensis* (Klich, unpublished data) that produce aflatoxin. A significant body of literature has been generated on the chemistry, enzymology, and genetics of aflatoxins mainly through the study of both *A. flavus* and *A. parasiticus*. These two fungi are most commonly associated with preharvest aflatoxin contamination of food and feed crops [reviewed in Cary et al. (2000)].

Sterigmatocystin, the penultimate precursor to aflatoxin is produced by a number of fungi including *A. nidulans*, which has served as the model system for the study of sterigmatocystin biosynthesis. Molecular genetic studies have shown that the genes for both the aflatoxin and sterigmatocystin biosynthetic pathways are clustered (Brown et al. 1996; Trail et al. 1995; Yu et al. 1995). A high degree of conservation exists between the aflatoxin clusters in *A. flavus* and *A. parasiticus*. The order of the genes on the pathway and their direction of transcription are identical. In addition, there is a high degree of sequence conservation (>95%) at both the nucleotide and amino acid level (Yu et al. 1995). Comparison

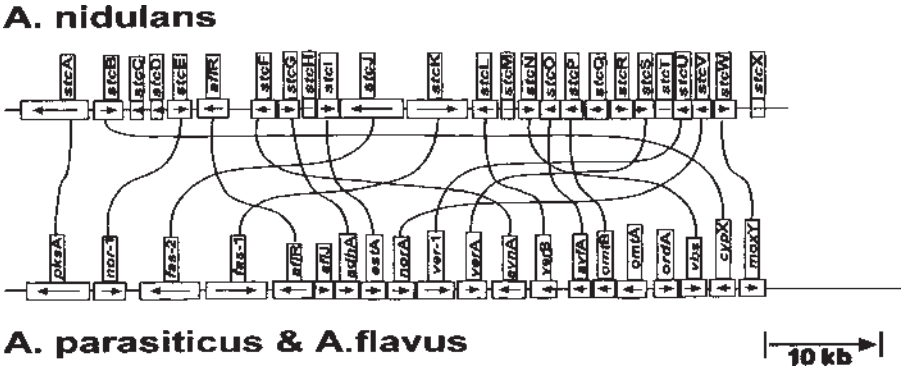

Figure 1 Comparison of the organization of genes in the aflatoxin biosynthetic pathway gene cluster from *Aspergillus flavus* and *A. parasiticus* and in the sterigmatocystin pathway cluster from *A. nidulans*. Arrowheads indicate the direction of transcription (adapted from information in Brown et al. 1996; Cary et al. 2000).

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of the aflatoxin cluster to the *A. nidulans* sterigmatocystin gene cluster shows a high degree of similarity with respect to gene function and structure though sequence homology, but the order of the genes on the clusters between these *Aspergillus* species is not as highly conserved (Figure 1). DNA sequence analysis and transcript mapping of mRNAs produced under conditions conducive to aflatoxin formation has identified as many as 23 genes covering a span of approximately 75 kb of the *A. parasiticus* genome that have been proven or are believed to be involved in aflatoxin biosynthesis (Figure 1). These techniques were also used to identify 25 co-regulated transcripts covering approximately 60 kb of the *A. nidulans* genome that are responsible for the biosynthesis of sterigmatocystin (Brown et al. 1996; reviewed in Cary et al. (2000)). A region of DNA from the dothistromin toxin-producing pine pathogen, *Dothistroma pini*, was reported to contain aflatoxin-like genes including *dotA* that had 80% amino acid identity to *A. parasiticus ver-1* (Bradshaw et al. 2002).

A number of the genes present on both the aflatoxin and sterigmatocystin gene clusters have yet to have their exact functions elucidated. With respect to aflatoxin biosynthesis, examples of these include; *afl*T, believed to encode a transporter for excretion of aflatoxin from the fungal mycelia (P.-K. Chang, unpublished data); *afl*J, located adjacent to *aflR* does not demonstrate similarity to any known genes and appears to modulate expression of pathway genes (Meyers et al. 1998); *est*A (a homolog of *A. nidulans stc* I), proposed to encode an esterase involved in the conversion of versiconal hemiacetal acetate to versiconal (J. Cary, unpublished data); two dehydrogenases, *nor*A and *nor* B, believed to be involved in the conversion of norsolorinic acid to averantin and the production of norsolorinic acid from noranthrone respectively (Cary et al. 1996; J. Cary and D. Bhatnagar et al., unpublished results). Additionally, three *A. parasiticus* genes, *cyp*A, *cyp* X, and *mox* Y, present at each end of the gene cluster (Figure 1) demonstrate homology to monooxygenases (Yu et al. 2000). Expression of all of the above mentioned genes is regulated in a manner similar to that of known aflatoxin biosynthetic genes and determination of their functions is being pursued.

Common to both the aflatoxin and sterigmatocystin gene clusters is the presence of a gene, designated *aflR*, that encodes a zinc binuclear cluster-type, sequence specific DNA-binding protein that has been shown to be necessary for expression of the genes in both clusters (Chang et al. 1995b; Ehrlich et al. 1998; Yu et al. 1996). In *A. nidulans*, binding studies using a recombinant form of the AflR protein and either a region of the *stc* U promoter or oligonucleotides based on sequence from within the promoter fragment demonstrated binding of AflR to the palindromic sequence 5′-TCGN$_5$CGA-3′ (Fernandes et al. 1998). The validity of this binding site was confirmed in a survey of 11 aflatoxin pathway genes from *A. parasiticus* using the recombinant AflR with oligonucleotides designed to the upstream regions of the 11 genes (Ehrlich et al. 1999). Based on these studies and data acquired from additional pathway genes, the consensus AflR binding sequence was determined to be 5′-SCGSWNNSCGR-3′.

2.2 Trichothecenes

Trichothecenes consist of a family of over 60 sesquiterpenoid compounds produced by a number of genera of filamentous fungi. Perhaps the best studied of the trichothecene producing fungi is *Fusarium* which is responsible for trichothecene contamination of grains such as maize, wheat, barley, and rye [reviewed in Proctor (2000)]. Examples of trichothecenes produced by *Fusarium* species include diacetoxyscirpenol (DAS), deoxynivalenol (DON), nivalenol (NIV), and T-2 toxin, while the more structurally complex macrocyclic trichothecenes are produced by fungal genera such as *Myrothecium*, *Stachybotrys*, and *Trichothecium*. Ingestion of trichothecene contaminated foods and feeds is associated with several human and animal diseases including acute toxicoses that can lead to death (Sharma and Kim 1991). Though the exact reason for the toxic effects attributable to trichothecene poisoning is not well understood it is believed to be the result of inhibition of protein synthesis that induces the disease symptoms.

The biosynthesis of trichothecenes begins with the cyclization of farnesyl pyrophosphate by trichodiene synthase to produce trichodiene. Trichodiene then undergoes an ordered series of oxygenation, isomerization, and esterification reactions leading to the formation of the various members of the trichothecenes [reviewed in Proctor (2000)]. A total of 10 cluster genes (TRI3-12) have been identified and characterized in *Fusarium sporotrichioides* that appear to be involved in trichothecene biosynthesis [reviewed in Cary et al. (2001)]. TRI101 has also been shown to be involved in trichothecene biosynthesis but it is not linked to the cluster (McCormick et al. 1999). Recently, an additional gene, TRI13, was also mapped to the cluster and shown to encode a protein with similarity to cytochrome P450 monooxygenases (Brown et al. 2002). Disruption studies showed that theTRI13 gene product oxygenates the C-4 position of calonectrin to yield 3,15-diacetoxyscirpenol (3,15-DAS). Further studies have shown that disruption of the TRI7 gene results in accumulation of HT-2 toxin that differs from T-2 toxin by an acetyl group at C-4 indicating that TRI7 is an acetyltransferase (Brown et al. 2001). Gene disruption and precursor feeding experiments have established that TRI8 is a C-3 esterase (McCormick and Alexander 2002). TRI8 mutants of *F. graminearum* no longer produced 15-acetyldeoxynivalenol but accumulated 3,15-DAS and other prescursors while *F. sporotrichioides* TRI8 mutants accumulated 3-acetyl T-2 toxin.

TRI101, a C-3 acetyltransferase, is the only trichothecene gene thus far characterized that is not linked to the gene cluster (Jurgenson et al. 2001; Kimura et al. 1998a, b; McCormick et al. 1999). Interestingly, this gene product was demonstrated to play a role in self protection as FsTRI101 deletion strains are significantly reduced in growth on

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trichothecene containing media compared to wild-type (McCormick et al. 1999).

At present, 3 of the 12 cluster genes (TRI6, TRI9, and TRI10) appear to be involved in regulating the levels of trichothecene production. TRI6 encodes a Cys_2His_2 type zinc-finger protein that is necessary but not sufficient for the expression of trichothecene biosynthetic pathway genes (Hohn et al. 1999). Disruption of TRI6 abrogates trichothecene biosynthesis. TRI6 was shown to bind in the promoter regions of nine pathway genes at the minimum consensus site 5′-YNAGGCC-3′. Though the exact role of TRI9 has yet to be determined, deletion of this gene in *F. sporotrichioides* resulted in strains making two fold more T-2 toxin relative to wild-type (D. Brown, unpublished results). This would indicate a possible regulatory role for TRI9. TRI10 also has an apparent role in regulation of TRI gene expression though like TRI9, it does not display any significant identity to proteins of known or predicted function (Tag et al. 2001). Disruption of TRI10 in *F. sporotrichioides* abolished T-2 toxin production and significantly decreased transcript accumulation of four trichothecene genes. Interestingly, one of these four TRI genes was TRI6 indicating that TRI10 acts upstream of TRI6 and is necessary for full expression of TRI pathway genes as well as genes that comprise a primary metabolic pathway upstream of the trichothecene gene cluster.

A comparison of the TRI gene coding sequences from *F. sporotrichioides* (FsTRI) and a DON-producing strain of *F. graminearum* (FgTRI) showed a high degree of identity between the two at both the nucleotide and predicted amino acid level (Brown et al. 2001). Both gene clusters spanned about a 26 kb region (Figure 2). The organization of the genes within their clusters was the same as was their direction of transcription. Sequence analysis of the FgTRI7 and FgTRI13 indicated that these genes are nonfunctional. This was expected, as neither TRI product is required for the biosynthesis of DON. In contrast, both of these genes appear to be functional in a NIV-producing strain of *F. graminearum* (Brown et al. 2002; Lee et al. 2002). NIV differs from DON in that it is hydroxylated in the C-4 position. It should be noted that there are a number of enzymatic steps in trichothecene biosynthesis for which the encoding gene has not been identified indicating that perhaps some of the TRI biosynthetic genes are not present on the cluster.

Production of the structurally more complex macrocyclic trichothecenes such as roridin E, verrucrin, and baccharinoid B7 is most commonly associated with members of the genus *Myrothecium*. The macrocyclic trichothecenes exhibit about 10-fold more toxicity than the *Fusarium* trichothecenes. Screening of a *M. roridum* cosmid library with a *F. sporotrichioides* TRI5 gene probe identified three overlapping cosmid clones that were used for gene isolation and cosmid mapping studies (Trapp et al. 1998). This work led to the identification in *M. roridum* of homologs for the *F. sporotichioides* TRI5 (MrTRI5), TRI4 (MrTRI4), and TRI6 (MrTRI6) genes [reviewed in Cary et al. (2000)]. It should be noted that the *M. roridum* trichothecene gene cluster is organized differently than the *Fusarium* trichothecene gene cluster so far characterized (Trapp et al. 1998).

2.3 Penicillins and Cephalosporins

Penicillins are β-lactam antibiotics that are produced exclusively by a few filamentous fungi, most notably *A. nidulans* and *P. chrysogenum*. The β-lactam antibiotics, cephalosporins, are produced by the fungus *Acremonium chrysogenum* (syn. *Cephalosporium acremonium*) as well as a number of bacteria [reviewed in Brakhage (1998)]. The biosynthesis of penicillins and cephalosporins has the first two steps in common. The first step involves the condensation of amino acid precursors to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). This reaction is carried out by a single, multifunctional enzyme, ACV synthase (ACVS), that is encoded by the gene *acv*A (*pcb*AB). The second step involves production of the bicyclic ring structure (the β-lactam ring fused to the thiozolidine ring) and is catalyzed by isopenicillin N synthase (IPN) which is encoded by *ipn*A (*pcb*C). At this point, the penicillin and cephalosporin biosynthetic pathways diverge. In the penicillin pathway, an acyl CoA:IPN acyltransferase encoded by *aat*A (*pen* DE), produces penicillin while the products of *cef*D, *cef*EF, and *cef*G result in the production of cephalosporin [reviewed in Brakhage (1998)].

The genes for both penicillin and cephalosporin biosynthesis are clustered. Penicillin gene clusters have been identified in *A. nidulans*, *P. notatum*, *P. griseofulvum*, *P. nalgiovense*, and *P. chrysogenum* (Laich et al. 2002).

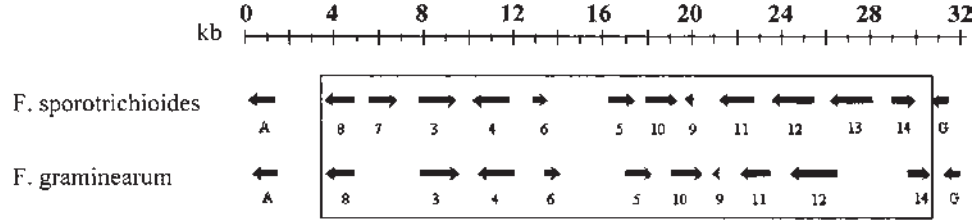

Figure 2 Genomic organization of the trichothecene biosynthetic gene cluster in *F. sporotrichioides* and *F. graminearum*. Arrowheads indicate the direction of transcription. Numbers underneath each arrow refer to the specific *TRI* gene (courtesy of D. Brown).

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In the penicillin gene cluster the *acv*A, *ipn*A, and *aat* genes are localized within a region of approximately 15 kb, while in *A. chrysogenum* the *acv*A and *ipn*A genes (early cluster) also reside within about a 15 kb region of chromosome VII (Gutierrez et al. 1999; MacCabe et al. 1990). In all the fungi the *acv*A gene is divergently transcribed from the *ipn*A gene while the *aat*A gene is transcribed in the same direction as *ipn*A. The cephalosporin pathway-specific genes, *cef*EF and *cef*G (late cluster), are located on a separate chromosome (chromosome I) in *A. chrysogenum* and are separated by a 938 bp intergenic region from which both genes are divergently transcribed (Gutierrez et al. 1992; 1999). The location of the *cef*D gene has yet to be determined in *A. chrysogenum*.

A number of different nutritional and developmental factors have been identified that regulate the biosynthesis of penicillins and cephalosporins. Due to the availability of mutants and ease of genetic manipulation, the majority of regulatory studies have focused on the penicillin biosynthetic pathway of *A. nidulans* [reviewed in Brakhage (1998); Martin (2000)]. Penicillin biosynthesis has been shown to be under the control of global-regulators of nitrogen and carbon metabolism, as well as pH (reviewed in Martin 2000). Due to the multi-faceted regulation of penicillin biosynthesis, penicillin gene expression is the result of a complex and cooperative interaction between these global-acting regulatory DNA-binding proteins. In addition, it has been found that high-level penicillin production in some strains of *P. chrysogenum* is due to the presence of amplified tandem repeats of the penicillin gene cluster [reviewed in Martin (2000)].

2.4 Gibberellins

Gibberellins are a large family of diterpenoid hormones that are produced by green plants, fungi, and bacteria. The rice pathogen *Gibberella fujikuroi* (mating population C) produces large amounts of gibberellic acid and it is a main commercial source of the bioactive gibberellins, particularly GA_3. Gibberellin (GA) biosynthesis follows the early steps in the general isoprenoid biosynthetic pathway until it branches from synthesis of carotenoids at geranylgeranyl-diphosphate (GGDP) [reviewed in Tudzynski (1999)]. The various gibberellins are then produced via a complex series of oxidations and hydroxylations. At least six genes have been shown to be specifically involved in GA biosynthesis and are present as a cluster spanning about 17 kb on chromosome 4 (Figure 3) (Tudzynski and Holter 1998). These genes encode the bifunctional *ent*-copalyl diphosphate synthase (cps/ks), a GA-specific geranylgeranyl diphosphate synthase (ggs2), and four cytochrome P450 monooxygenases (Tudzynski et al. 2001). The biosynthesis of fungal GAs requires 13 enzymatic steps; therefore it was expected that some of the known biosynthetic genes would be multifunctional, and the *P450-1* gene was found to catalyze four oxidation steps from *ent*-kaurenoic acid to GA14 via GA12-aldehyde (Rojas et al. 2001). Additionally, it was found that the *P450-4* gene encoded a multifunctional *ent*-kaurene oxidase, catalyzing all three oxidation steps between *ent*-kaurene and *ent*-kaurenoic acid (Tudzynski et al. 2001). A seventh gene, orf 3, upstream of *P450-4* has been identified but its function has yet to be identified (Voss et al. 2001). An MFS-transporter gene, *smt*, was identified upstream of *orf 3* but it was shown not to be involved in secretion of GA.

Very little is known about the regulation of gibberellin production at the molecular genetic level. To date a positive-acting, GA pathway-specific regulator protein has not been identified in *G. fujikuroi*. The success of complementation experiments using a mutant strain that has a large deletion on chromosome 4 resulting in the loss of the entire GA gene cluster indicates that regulatory factors required for expression of GA biosynthetic genes are not located within the cluster. Studies have shown that production of GA is regulated by nitrogen (Tudzynski et al. 1999). AreA-GF of *G. fujikuroi* is a homolog of the global nitrogen regulator AreA of *A. nidulans*. Disruption of *are*A-GF was shown to significantly reduce GA production and complemention of the *are*A-GF mutant with a wild-type *are*A-GF restored the GA production. Northern blot analysis performed on five of the GA pathway genes showed that transcript levels are drastically increased under conditions of ammonium limitation (gibberellin inducing), which would be expected from the action of a positive-acting regulatory factor like AreA-GF (Tudzynski and Holter 1998). Potential GATA binding motifs for AreA-GF have been identified upstream of GA biosynthetic genes.

2.5 Fumonisins

Fumonisins are polyketide mycotoxins that are produced by a number of *Fusarium* species within section Lesiola and some isolates of *F. oxysporum* (Desjardins and Proctor 1999). Of particular concern is the widespread infection of maize by the fungus, *F. verticillioides* (*G. moniliformis*, syn. *G. fujikuroi*

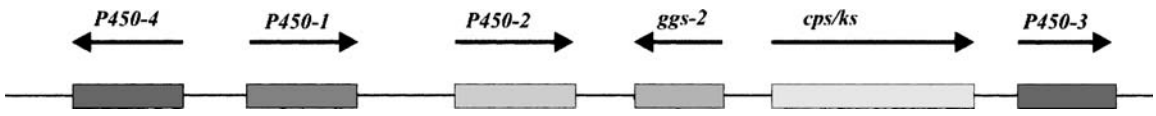

Figure 3 Gibberellin biosynthetic gene cluster in *G. fujikuroi*. Arrowheads indicate the direction of transcription (courtesy of B. Tudzynski).

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mating population A). These toxins have been shown to cause or are associated with a number of diseases in animals and humans including cancer following consumption of contaminated grain (Marasas 1996). Fumonisins consist of a linear 19- or 20-carbon backbone that is substituted at various positions with hydroxyl, methyl, and tricarballylic acid moieties and an amine group at C-1 or C-2 (reviewed in Proctor 2000). Until recently it was not known if fumonisins were the product of either polyketide or fatty acid biosynthesis. However, disruption and complementation analysis of a cloned *F. verticillioides* gene, *FUM5*, encoding a polyketide synthase demonstrated that fumonisins are a product of polyketide biosynthesis (Proctor et al. 1999).

Classical genetic analyses of fumonisin (*Fum*) mutants of *F. verticillioides* indicated that the genes for fumonisin biosynthesis are closely linked on chromosome 1 (Desjardins et al. 1996). Complementation analysis of *Fum* mutants with cosmid DNA harboring the *FUM5*gene supported the meiotic analyses indicating that the genes involved in fumonisin biosynthesis are clustered (Proctor et al. 1999). The structure of FB_1 suggests that there may be as many eight enzymes required for toxin synthesis. Recent work has identified the presence of an additional 14 genes immediately downstream of *FUM5* and evidence suggests that they are involved in fumonisin biosynthesis (Proctor et al. 2001; Seo et al. 2001). Four of these genes (*FUM6*, *FUM7*, *FUM8*, and *FUM9*) have been studied in more detail. Disruption of *FUM6* and *FUM8* blocked fumonisin production and the expression analysis of all four of these *FUM* genes correlated with fumonisin production in *F. verticillioides*. Based on comparison of their predicted amino acid sequences with those in the GenBank database, three of the four gene product's predicted activities are consistent with activities expected to be involved in fumonisin biosynthesis. *FUM6* appears to encode a unique P450 monooxygenase fused to an NADPH-dependent P450 reductase that may be involved in the hydroxylation of the fumonisin backbone. The *FUM8* product displayed similarity to class-II $''$-aminotransferases indicating that it may catalyze the condensation of alanine and a linear polyketide that is believed to constitute the fumonisin backbone. Though *FUM7* and *FUM9* have not been disrupted, sequence analysis indicates that their products could theoretically be involved in toxin synthesis. The predicted *FUM9* protein demonstrated a low level of similarity to oxoglutarate-dependent dioxygenases and therefore may be involved in the hydroxylation of the fumonisin backbone. The predicted *FUM7* protein showed similarity to type III alcohol dehydrogenases and was hypothesized to function as a carbonyl reductase capable of reducing the carbonyl group at C-3 of the fumonisin backbone.

To date, the predicted translation products of the 14 genes downstream of *FUM5* failed to identify a gene that may encode a regulatory protein involved in fumonisin biosynthesis. However, one of the ORFs did share similarity with known transporter genes (Proctor et al. 2001).

2.6 Melanins

A number of fungi produce melanins which are the black or near-black pigments formed by oxidative polymerization of phenolic compounds produced by the dihydroxynaphthalene (DHN)-melanin pathway. Melanin has been shown to be a virulence factor in plant, animal, and human pathogenic fungi and it also functions in survival and longevity in nature of fungal propagules [reviewed in Butler and Day (1998)]. In addition to its function in UV-tolerance, studies of melanin mutants of *Alternaria alternata* also showed that melanins play a role in conidial development (Kawamura et al. 1999). Melanin biosynthesis initiates through the action of a polyketide synthase that converts acetate to the intermediate compound 1,3,6,8-tetrahydroxy-naphthalene (T4HN), which is subsequently reduced by scytalone reductase to scytalone. Dehydration of scytalone forms 1,3,8-trihydroxynaphthalene (T3HN) that is then converted to 1,8-dihydroxynaphthalene (DHN) after an additional reduction and dehydration step. DHN is then polymerized and oxidized to yield melanin.

The genes involved in melanin biosynthesis are clustered in some fungi while in others they are not. Complementation of *A. alternata* melanin-deficient mutants with cosmid DNA allowed the identification of an approximately 30 kb region of the *A. alternata* genome that harbored three genes encoding the polyketide synthase (*ALM*), scytalone dehydratase (*BRM1*), and T3HN reductase (*BRM2*) involved in melanin biosynthesis. Subsequent gene disruption experiments confirmed the function of these genes in melanin biosynthesis (Kimura and Tsuge 1993). Expression of these three genes was synchronous with the onset of mycelial melanization. A developmentally regulated gene cluster involved in pigment biosynthesis has been characterized in the human opportunistic pathogen, *A. fumigatus*, which produces a blue–green conidial pigment (Tsai et al. 1999). Sequence and gene disruption analysis of DNA from a cosmid clone complementing an *A. fumigatus* conidial color mutant identified six genes (*alb1*, *arp2*, *arp1*, *abr1*, *abr2*, and *ayg1*) within a 19 kb region that were involved in pigment biosynthesis. All six genes were developmentally regulated, being expressed during conidiation. The gene products of *alb*1, *arp*1, and *arp*2 demonstrated significant identity at the amino acid level with polyketide synthases, scytalone dehyrdratases, and T3HN reductases of brown and black fungi, respectively. Disruption of the *alb*1 gene of *A. fumigatus* significantly reduced virulence in a murine model just as disruption of melanin genes in plant pathogenic fungi severely reduces virulence (Tsai et al. 1998). Though database-homology searches did not identify any proteins with similarity to the deduced protein encoded by *ayg1*, *in vitro*, cell free assays using extracts of *A. oryzae* expressing *ayg1* combined with genetic complementation experiments showed that Ayg1 catalyzes the formation of 1,3,6,8-THN by a novel chain length-shortening of a heptaketide precursor formed by the Alb1 PKS (Tsai et al. 2001). Similarity searches of sequence databases showed that of the other two genes, *abr*1 encoded

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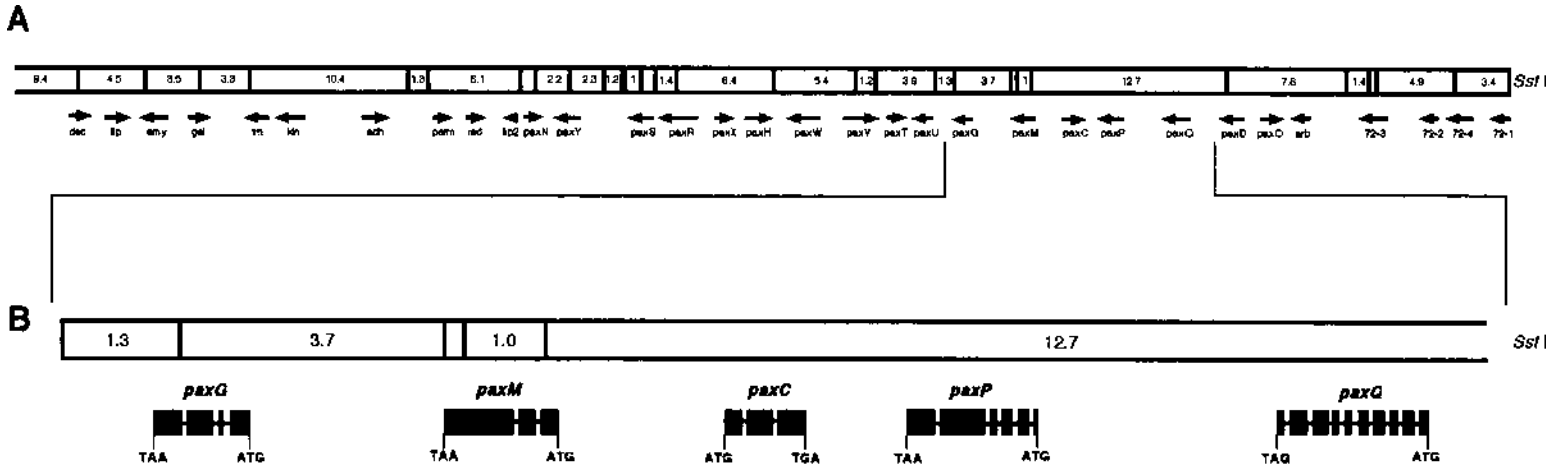

Figure 4 Genomic organization of the paxilline biosynthetic gene cluster and flanking genes in *P. paxilli*. A) Physical map of the paxilline gene cluster depicting sizes of *Sst* I restriction fragments. Arrowheads indicate the direction of transcription. B) Enlargement of the DNA region spanning *paxG* to *paxQ*. Shaded boxes indicate exons within each *pax* gene (courtesy of B. Scott).

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a putative multicopper oxidase and *abr*2 a putative laccase (Tsai et al. 1999). Genes homologous to the *A. alternata* DHN-melanin biosynthetic genes have been identified in plant pathogenic fungi such as *Colletotrichum lagenarium* and *Magnaporthe grisea* (Chumley and Valent 1990; Kubo et al. 1996a, b). However, none of the genes have been shown to be closely linked, though the expression of the *C. lagenarium* genes appear to be developmentally controlled as in the *A. alternata* melanin gene cluster (Tsuji et al. 2000). A melanin pathway-specific regulatory protein has yet to be identified that is linked to the melanin gene cluster in *A. alternata* or *A. fumigatis*.

2.7 Paxilline

Paxilline is a tremorgenic, indole-diterpenoid, mycotoxin produced by the filamentous fungus, *Penicillium paxilli* (Cole et al. 1974). Though little is known about the biosynthesis of these mycotoxins, geranylgeranyl pyrophosphate (GGPP) and indole are presumed to be precursors. Utilizing plasmid-tagged mutagenesis and REMI-mutagenesis techniques, a number of paxilline negative, deletion mutants of *P. paxilli* have been isolated and characterized (Young et al. 1998). Analysis of *P. paxilli* genomic DNA flanking and including the deleted regions of the mutants identified a cluster of 17 ORFs with similarities to prenyltransferases and monooxygenases (Young et al. 2001). The boundaries of the cluster have been defined to a size of about 50 kb on chromosome Va (Figure 4). Targeted disruption of a GGPP synthase (*paxG*) confirmed that this gene was required for toxin synthesis but not for primary metabolism. Additional ORFs were identified with sequence similarities to two FAD-dependent monooxygenase (*paxM* and *paxN*), a prenyltransferase (*paxC*), two cytochrome P450 monooxygenases (*paxP* and *paxQ*), a dimethylallyltryptophan synthase (*paxD*), a dehydrogenase (*paxH*), a transporter (*paxT*), and an oxidoreductase (*paxO*). Deletion of *paxP* and *paxQ* resulted in accumulation of the metabolites paspaline and 13-desoxypaxilline respectively (McMillan et al. unpublished data). An additional 5 ORFs (*paxU,V,W, X*, and *Y*) were identified that could not be correlated with a function based on sequence similarities to known proteins. RT-PCR analysis demonstrated that the expression of *paxG*, *paxM*, and *paxP* increased dramatically with the onset of paxilline biosynthesis (Young et al. 2001).

Two genes (*paxR*and *paxS*), residing in the cluster, demonstrated similarity with Zn(II)2Cys6 binuclear DNA-binding proteins known to be involved in the positive regulation of secondary metabolic pathways. Though further analysis will be needed to confirm their roles as transcriptional regulators, this would suggest that the mechanism of regulation of paxilline biosynthesis may differ from that of aflatoxin and sterigmatocystin biosynthesis that have only one positive-acting transcriptional regulator within their respective gene clusters.

3 HOST-SPECIFIC TOXINS

3.1 HC-Toxin

HC-toxin is an epoxide-containing cyclic tetrapeptide that is a critical virulence determinant in the pathogenic interaction between *Cochliobolus carbonum* and maize (Walton 1996). HC-toxin is a potent inhibitor of histone deacetylaces from maize and other organisms (Ransom and Walton 1997). Classical genetic analysis of HC-toxin producing (Tox 2^{+}) isolates of *C. carbonum* indicated that production was under control of a single locus, designated *TOX2*. Further molecular analysis showed that *TOX2* is highly complex consisting of at least seven duplicated and co-regulated genes localized within a region of about 600 kb of a single 3.5 Mb chromosome in strain SB111 (Ahn et al. 2002). These genes include, *HTS1*, encoding a tetrapartite cyclic peptide synthetase; *TOXA*, encoding an HC-toxin membrane transporter of the major facilitator superfamily; *TOXC*, encoding a fatty acid synthase beta subunit believed to be responsible in part for the synthesis of the decanoic acid backbone of 2-amino-9,10-epoxi-8-oxodecanoic acid (Aeo) moiety of the cyclic tetrapeptide [reviewed in Walton et al. (1998)]. Although its role in HC-toxin biosynthesis has not been established, *TOXD* is predicted to encode a dehydrogenase based on sequence similarity to that of *lovC*, a gene required for lovastatin biosynthesis in *A. terreus*. Targeted disruption of *TOXF* and *TOXG* abolished toxin production and pathogenicity (Cheng and Walton 2000; Cheng et al. 1999). The deduced amino acid sequence of *TOXF* has moderate homology to many known branched-chain, amino acid transaminases suggesting that *TOXF* may function to aminate a precursor of the Aeo-moiety of HC-toxin (Cheng et al. 1999). *TOXG* is predicted to encode an alanine racemase (Cheng and Walton 2000).

TOXE encodes a novel regulatory protein involved in HC-toxin biosynthesis. The *TOXE* protein has a bZIP basic DNA binding domain but no discernable leucine zipper characteristic of other bZIP transcription factors. *TOXE* has four ankyrin repeats at its carboxy-terminus also found in other regulatory proteins but to date these repeats have never been associated with a bZIP-type DNA binding domain. It was determined that *TOXE* is a DNA-binding protein that recognizes a consensus ten base motif (ATCTCNCGNA), "tox-box," present in the promoters of all known *TOX2*genes (Pedley and Walton 2001). *TOXE* expression was shown to be required for expression of all the other toxin genes except *HTS1* (Ahn and Walton 1998). None of the above mentioned *TOX2* genes are present in HC-toxin nonproducing (Tox2^{-}) strains.

3.2 AK-Toxin

The Japanese pear pathotype of *A. alternata* causes black spot of Japanese pear by producing the host-specific toxin, AK-toxin (Otani et al. 1985). Utilizing restriction

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enzyme-mediated integration (REMI) mutagenesis of *A. alternata* pear pathotype strain 15A resulted in the isolation of fungal transformants that produced no AK-toxin (Tanaka et al. 1999). Two genes, AKT1 and AKT2, were identified within a 5 kb region of the genome which when disrupted resulted in loss of AK-toxin production and pathogenicity. Disruption of either gene resulted in isolation of transformants that were no longer pathogenic and failed to produce the AK-toxin precursor 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid. AKT1 demonstrated homology with carboxyl-activating enzymes and it was theorized that its product activates an earlier precursor of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid for further modification by other enzymes involved in AK-toxin production. AKT2 demonstrated no similarity to known proteins. Interestingly, homologs of both AKT1 and AKT2 were detected in Southern blots of DNA from *A. alternata* tangerine (ACT-toxin) and strawberry (AF-toxin) pathotypes but not in other pathotypes or non-pathogenic strains of *A. alternata* (Masunaka et al. 2000; Tanaka et al. 1999). All three of these pathotypes share the common precursor moiety 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid suggesting the possibility of horizontal transfer of these genes between these pathotypes (Tanaka et al. 1999). Additionally, it was found that wild-type strains carried additional, non-functional copies of these two genes.

Two ORFs, designated AKTR and AKT3, were also shown to be involved in AK-toxin production (Tanaka and Tsuge 2000). AKTR encodes a putative zinc binuclear cluster DNA-binding protein with an internal leucine zipper domain. AKT3 encodes a protein that has similarity to members of the hydratase/isomerase enzyme family. As with AKT1 and AKT2, there were multiple, nonidentical copies of AKTR and AKT3 present. All of the genes were believed to be present on the same 4.1 Mb chromosome. These results indicate a high level of structural and functional complexity with respect to the genomic region controlling AK-toxin biosynthesis. No information has been gleaned as to the molecular mechanisms controlling AK-toxin biosynthesis.

4 SIGNIFICANCE OF SECONDARY METABOLIC GENE CLUSTERS

4.1 Regulation of Gene Cluster Expression

A primary advantage of gene clustering may be for the purpose of coordinated gene expression. Clustering of genes allows regulatory elements to be shared, as is seen with the promoter regions of the divergently transcribed *acvA-ipnA* genes in the penicillin gene cluster (see Section 2.3) and the *niiA–niaD* genes of *Aspergillus* sp. nitrate assimilation gene cluster (Chang et al. 1996). Though sharing of regulatory regions might be part of the reason for clustering it does not account for co-regulation of genes found in larger gene clusters containing ten or more genes, many of which are not divergently transcribed. There is evidence that gene clustering may influence gene regulation through modulation of localized chromatin structure. Transformation of *A. parasiticus* with the promoter region of the aflatoxin biosynthetic genes *ver-1A* or *nor-1* fused to a GUS reporter gene showed that the site of integration within the fungal genome affects gene expression (Chiou et al. 2002; Liang et al. 1997). Homologous integration of the reporter constructs within the aflatoxin gene cluster did not significantly affect GUS expression while integration outside the cluster at the *niaD* or *pyrG* locus resulted in near undetectable levels of GUS activity. It was hypothesized that this position-dependent gene expression may be due to the presence of *cis*-acting enhancer elements (Chiou et al. 2002). The presence of enhancer elements in the *nor-1* and *ver-1* gene regions has yet to be proven. Similar position-dependent effects have been observed for expression of ectopically integrated genes of the SpoC1 gene cluster in *A. nidulans* (Miller et al. 1987). Close association of pathway biosynthetic genes does not appear to be a prerequisite for coordinated gene expression as demonstrated by the melanin biosynthetic pathway of *C. lagenarium* (Kubo et al. 1996b) and *M. grisea* (Chumley and Valent 1990), and the nitrogen assimilation pathway of *N. crassa* (Exley et al. 1993) and *G. fujikuroi* (Tudzynski et al. 1996), in which the genes are unlinked yet regulated in a coordinate fashion.

Continued studies of mechanisms controlling the regulation of gene expression within secondary metabolic gene clusters, such as the role of cluster chromatin structure and/or enhancer elements, may provide supportive evidence for any advantage that clustering imparts to production of the secondary metabolite.

4.2 Evolutionary Significance

Other than potential regulatory advantages, gene clusters may also have arisen through the horizontal genetic transfer of secondary metabolic pathways between prokaryotes and fungi, and perhaps from fungi to fungi. This is most clearly demonstrated by the observation that the gene clusters for penicillin and cephalosporin biosynthesis from prokaryotes are also present in fungi. The fungal genes in the clusters for the synthesis of penicillin and cephalosporin contain features of the prokaryotic pathway genes, such as the absence of introns and high G + C content (Weigel et al. 1988) indicating horizontal transfer. Not as readily explained though is the fact that several of the metabolic pathways and the corresponding gene clusters seem to be unique to fungi, e.g., the mycotoxin and host-specific toxin biosynthetic gene clusters. And within gene clusters with similarity to prokaryotic systems, several genes seem to be of fungal origin, e.g., the *cef*G gene in cephalosporin C pathway of *A. chrysogenum* and the *aat* gene of the penicillin pathway of *A. nidulans* and *P. chrysogenum* (Mathison et al. 1993). This indicates that these genes were "recruited" from the fungal genome for the purpose of β-lactam biosynthesis, but why were they positioned so as to form part of a gene cluster?

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Again, this would suggest that clustering of genes is functionally important, perhaps reflecting that coordinate regulation of gene expression is imparted by localized chromatin structure. Therefore, the possibility does exist that due to evolutionary pressures, individual genes (or in some cases significant parts of a cluster) were acquired by fungi from prokaryotes. But aside from the penicillin and cephalosporin genes there is very little evidence of horizontal transfer of pathway gene clusters from prokaryotes to fungi in nature.

Yet another plausible hypothesis put forth for evolutionary significance of gene clusters in fungi is that having acquired individual genes for specific enzyme activities from either prokaryotes (e.g., penicillin pathway genes; Weigel et al. 1988) or other fungi (Rosewich and Kistler 2000) by HGT, fungi organized these genes into clusters for specific metabolic functions. How these recruited genes eventually amalgamated into a gene cluster is not clear. Over the course of evolution these individual or small groups of genes may have been united via transpositions, reciprocal translocations, deletions, or other events causing chromosomal rearrangements that were maintained as a result of positive selective pressure. Perhaps the best evidence to date for transfer of a gene(s) between phylogenetically unrelated fungi would be with respect to the PEP gene cluster of the pea pathogen, *Nectria haematococca* (Han et al. 2001). The absence of the PEP cluster in nonpathogenic *N. haematococca* and other features of the cluster such as distinctive codon usage and the presence of transposable elements is suggestive of acquisition via horizontal transfer. The presence of PEP cluster gene homologs in strains of *F. oxysporum* pathogenic on pea but not present in strains of *F. oxysporum* pathogenic on other plants suggests possible horizontal transfer between two unrelated organisms that occupy the same ecological niche.

While the possibility of horizontal transfer of individual or small groups of genes is quite plausible, what are the chances for the transfer of an entire gene cluster, even between related fungal species? To date there is little evidence for horizontal transfer of a gene cluster between fungi. However, the frequency of gene cluster transfer between related or unrelated fungal species may actually be much higher than predicted, but detection of these events has been limited by the number of fungal samples obtained from nature for analysis.

Another hypothesis was put forth by Walton (2000) termed the "selfish cluster" hypothesis that states, "clustering has evolved and is maintained because it confers selective advantage to the cluster itself, and this selective pressure is distinct from the pressure that maintains the capacity to produce the secondary metabolite." A second codicil to this hypothesis states "the reason that clustering favors survival of secondary metabolic genes is because these kinds of genes depend, at least in part, on HGT for their dispersal and survival." Indeed, recent research has identified isolates in *Aspergillus* section *Flavi* of *A. pseudotamarii* (Ito et al. 2001), *A. bombycis* (Peterson et al. 2001) and one isolate of *A. ochraceoroseus* (section *Circumdati*) (Klich et al. 2000) that harbor aflatoxin gene clusters and are capable of producing aflatoxin.

Interestingly, the organization of the genes along the aflatoxin biosynthetic pathway cluster in *A. flavus* and *A. parasiticus* is quite different from that of the sterigmatocystin pathway cluster in *A. nidulans* that performs essentially the same function as the aflatoxin pathway (sterigmatocystin is a precursor of aflatoxins). What would be the evolutionary or physiological advantage to *A. nidulans* for this rearrangement? Perhaps *A. nidulans* is in the evolutionary process of dissolution of the original toxin pathway gene cluster. It may have already rid itself of the last two steps in aflatoxin synthesis (sterigmatocystin to *O*-methylsterigmatocystin to aflatoxin B_1). Keller and Hohn (1997) describe this process, "Gene cluster dissolution may in turn signal the first step in the eventual loss of the pathway or may simply reflect the lack of selection pressure required to maintain the cluster." Evidence exists for what is apparently an ongoing "break up" of fungal, host-specific toxin gene clusters. The HC-toxin gene cluster in some isolates of *C. carbonum* has apparently undergone a reciprocal translocation between chromosomes that resulted in at least one copy of TOXE being on a separate chromosome (Ahn et al. 2002). The absence of clustering of the genes for melanin biosynthesis in some fungi may also suggest dissolution of this gene cluster. The melanin biosynthetic pathway gene cluster is supposedly in the process of break-up to a differing extent in several fungi (Kubo et al. 1996a, b); with no apparent reason for this process. A consequence of this dissolution could be the utilization of one or more genes of the cluster towards related functions in other pathways.

These arguments will be substantiated as more metabolic pathways are characterized, and as one or more of these pathway clusters are detected in other related or unrelated fungi (i.e., aflatoxin and sterigmatocystin gene clusters). Additionally, with the discovery of aflatoxin pathway gene clusters in the non-aflatoxigenic *Aspergilli*, *A. oryzae*, and *A. sojae* (Section *Flavi*) (Chang et al. 1995a; Klich et al. 1995) and the presence of interrupted regulatory elements in an atoxigenic *Aspergillus* sp. (Geiser et al. 1998), more clues as to the reasons for the dissolution of pathway gene clusters may be uncovered, as well as to what may be the evolutionary significance of several of the metabolic pathway gene clusters.

5 CONCLUSIONS

With the characterization of a number of fungal secondary metabolic biosynthetic pathways it has become apparent that the physical organization of these pathways as clusters is more the rule than the exception. Characterization of these gene clusters has, in many cases, uncovered common components such as pathway specific regulatory and transporter genes. However, many questions still remain unanswered with respect to just what advantages these secondary metabolites and their associated gene clusters

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provide the fungus. Though many fungal secondary metabolic gene clusters may not appear to be required under conditions and over the narrow "window" of time that these fungi have been studied; it is quite possible that over the course of "evolutionary time" gene clusters have played an important role in the growth, survival, and evolution of these organisms. Much still remains to be elucidated with respect to the evolution of the complex mechanisms that (a) control expression of cluster genes and (b) were responsible for the acquisition, organization, and maintenance of the genes that constitute the cluster. There is conflicting evidence for any advantage of clustering on regulation of expression of the genes within the cluster. While the coordinate expression of unlinked melanin biosynthetic genes in *C. lagenarium* argues against any regulatory advantages of clustering; the significant reduction in expression observed in ectopically integrated aflatoxin biosynthetic genes in *A. parasiticus* supports the need for clustering as a means of localizing the biosynthetic genes within a transcriptionally-active region of DNA. It will be interesting to see if expression of genes in other secondary metabolic pathways are also subject to position-dependent regulation as observed in the aflatoxin pathway.

While evidence for HGT between fungi is largely anecdotal, there is a growing body of evidence that suggests HGT has played a role in the evolution of fungal secondary metabolic gene. As molecular phylogenetic techniques are refined and the body of fungal genome sequences expands there should be more information uncovered as to the incidences and mechanisms of HGT in fungi as well as its significance to the form and function fungal gene clusters.

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8

Application of Gene Cloning in Fungal Biotechnology

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1 INTRODUCTION

The major advantage of gene technology over a classical genetic approach is that gene technology allows cloning, characterization and modification of genes, regulatory regions, or any other kind of DNA sequences. Cloned sequences can be transferred at will, to new hosts that are more suitable for a detailed study of their function and regulation. Cloning techniques are of increasing value in the genomic era, particularly when new genome-programs are initiated almost every month, leading to the accumulation of enormous amounts of DNA sequence data. Analysis of this huge mass of sequence data requires further molecular genetic studies aimed at the functional analyses of the tremendous amount of raw sequence information. For example, the human genome program has resulted in the identification of the primary structure of the whole genome of *Homo sapiens*, but the function of more than 90% of the human genes are still unknown. An additional advantage of using gene technology is that it can be exploited in breeding programs, and the use of cloned genes allows for the most precise modification or transfer of a single trait, without affecting other properties of the breeding material.

The major purpose of cell biology research is to understand the relationships between biological processes and gene function, an approach that needs model organisms. Fungi are especially suitable as such model organisms for a number of reasons. They have relatively small and simply organized genomes. Some fungi, like most species of yeasts are unicellular organisms, whereas others, which are filamentous, can also be brought into genetically homogenous cultures, as their spores or appropriately manipulated vegetative cells are suitable for clonal propagation. Many fungi can be maintained indefinitely as haploid, mitotic lineages. These lineages can also be broken by initiating mating and sexual recombination. Almost all fungi can easily be manipulated by using molecular genetic techniques, including DNA-mediated transformation and site-specific mutagenesis.

Further advantages of fungi, as experimental organisms in biotechnology, are their outstanding metabolic versatility. Most filamentous ascomycetes, including the most favored subjects of molecular genetic studies, like members of the genera *Aspergillus*, *Neurospora*, or *Podospora*, can utilize a wide range of compounds as sole carbon or nitrogen sources and they are capable of degrading a number of highly polymerized compounds, like cellulose, pectin, or starch. At the same time, their intermediary metabolism shows striking similarities with that of the higher eukaryotes; metabolic pathways are, in some cases controlled by functionally interchangeable genes in fungi and higher eukaryotes. Finally, many fungi have been awarded the generally regarded as safe (GRAS) status, therefore they are regarded as favorable hosts for producing compounds, including novel type or recombinant molecules for human utilization.

Both basic molecular cell biology research and modern production biotechnology require the use of cloned genes. In this chapter we describe basic strategies of gene cloning and provide selected examples of utilizing cloned fungal genes in agricultural, industrial, and medical biotechnology.

2 CLONING STRATEGIES

Understanding fungal biology ultimately requires the functional characterization of the genes constituting the genome. A pre-requisite of functional characterization is the identification and cloning of these genes. The number of fungal genes that control cellular, physiological, and morphological processes amounts approximately to

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6000–9000 in a given species, and genome sizes range between 12 and 42 Mb (Kupfer et al. 1997).

Various approaches are available for cloning genes from fungi. The choice of the strategy to be followed depends on several aspects, including the experimental purpose and the applicability of techniques to the target organism. In this section an overview of the methodologies applied in gene cloning is presented. In terms of experimental strategies the methods are based on (a) selection for the phenotype attributable to the biological function of the gene, (b) detection of the biochemical activity of the gene product, (c) the structure of the gene, (d) transcriptional regulation of the gene, and (e) chromosomal position of the gene (Figure 1).

2.1 Cloning by Biological Function

When gene cloning is achieved by the identification of a phenotype attributable to the function of the product of the gene, two alternative strategies can be followed: complementation of mutants and generation of tagged insertional mutants.

2.1.1 Complementation of Mutants

If inactivation of a gene and the subsequent restoration of its function yield phenotypes that can be screened for, the gene responsible for this function can be cloned by complementing a mutant strain, which carries the inactivated gene. This requires the availability of appropriate stable mutants and an efficient gene transfer system. These requirements may not always be met in case of specific target organisms, therefore complementation experiments are frequently performed in heterologous hosts. *Saccharomyces cerevisiae* is the most frequently used host organism for this purpose. In complementation studies mutant strains are transformed with a gene library from the target organism, and complementing phenotypes indicative of the restoration of gene function are selected. Transformation vectors purified from these strains contain the gene(s) of interest. Depending on the type of library used, the vector may contain either a cDNA or a genomic fragment of the gene. In this latter case, especially when large insert libraries such as cosmid banks are used, identification of an open reading frame (ORF) responsible for the appropriate phenotype requires further subcloning and complementation experiments. This strategy

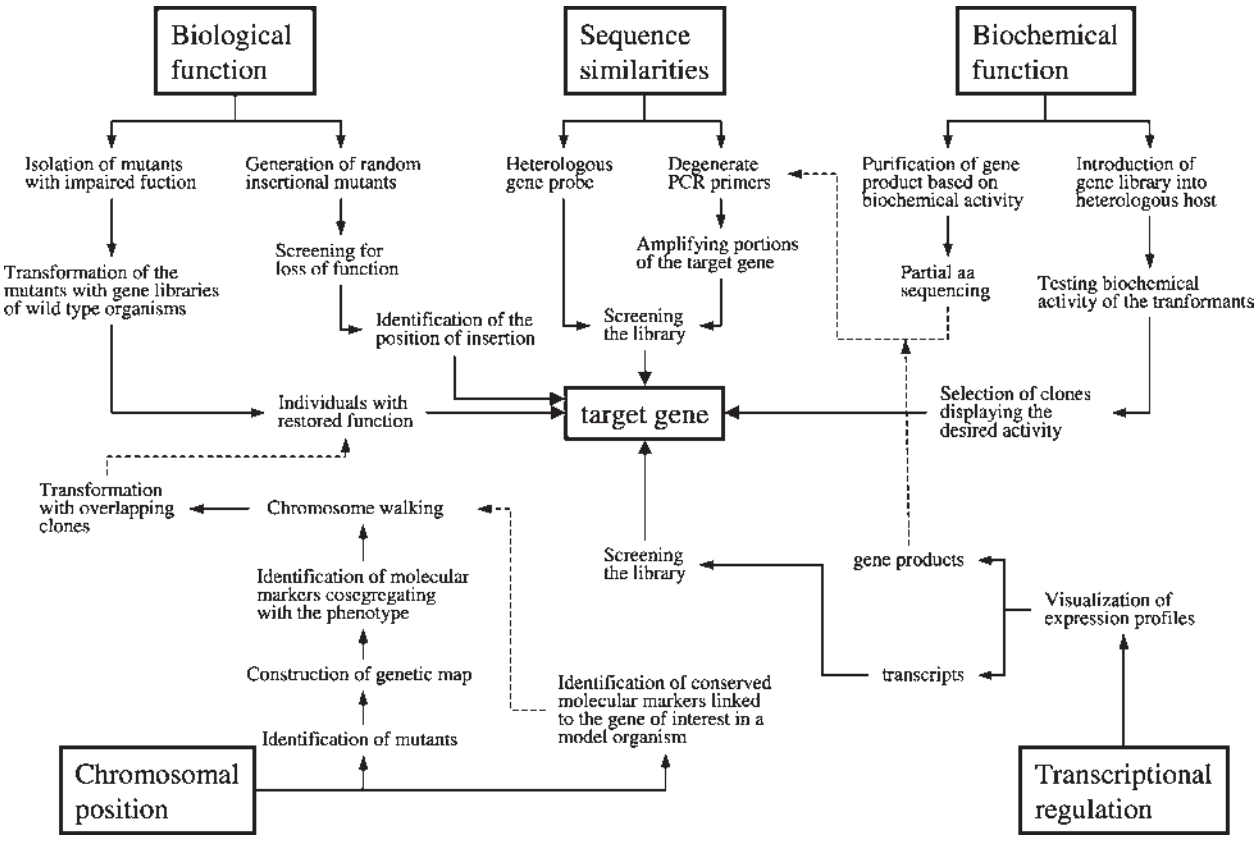

Figure 1 Schematic diagram of experimental approaches to gene cloning. Dashed lines indicate links between the different strategies. Further details are provided in the text. aa, amino acid.

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is most suited to isolation of a single gene responsible for a given biological function, and an example is the cloning of the *uac1* gene encoding an adenylate cyclase from *Ustilago maydis* by complementing a mutant strain incapable of yeast-like budding growth (Barrett et al. 1993). However, this approach is also suitable to clone a set of genes acting downstream of the inactivated gene in a biochemical pathway.

2.1.2 Insertional Mutagenesis

Insertional mutagenesis, often termed also as gene tagging, is an elegant way of generating loss-of-function mutants and allows the identification of genes responsible for specific biological functions. It can therefore be applied as an alternative gene cloning strategy from fungi. This approach is especially efficient if screening procedures suitable for testing large numbers of mutants are available. In general, a foreign sequence is introduced into the target organism and, as a consequence of chromosomal integrations, genes become inactivated. Ideally, integrations occur in a random fashion and in a single copy. The collection of transformants are subsequently screened for a specific mutant phenotype, usually for the loss of an ability of the target organism, e.g., impairment in pathogenicity. Regions, flanking the insertion sites are then identified by PCR-based techniques or plasmid rescue (Walser et al. 2001). This approach also requires efficient transformation systems for inserting sequences into the target organism.

(a) Restriction Enzyme Mediated Integration. The type of insertional mutagenesis, most widely used for studying fungal genes is restriction enzyme mediated integration (REMI). In REMI, fungal cells are transformed with a plasmid in the presence of a restriction endonuclease. The addition of the endonuclease facilitates integration of the vector DNA into the chromosome. The precise mechanism and critical parameters of the procedure are not fully understood, and the results obtained thus far are in part contradictory (Walser et al. 2001 and references therein), but in a number of experiments, the addition of restriction enzymes increased the transformation frequency and decreased the number of integrated copies of foreign DNA per transformant. This approach has mainly been used to study pathogenicity determinants in plant pathogens (Bolker et al. 1995; Sweigard et al. 1998).

(b) Signature Tagged Mutagenesis. Signature tagged mutagenesis (STM) is a method originally developed to identify genes essential for *in vivo* colonization of animal tissues by bacterial pathogens (Hensel et al. 1995). Separate insertional mutant pools are generated with tagged sequences, usually with transposons, and then a group of mutants tagged with distinct sequences are used to co-inoculate the host. In parallel, the same subpool of mutants is subjected to *in vitro* culturing. After an appropriate incubation time the quantitative representation of each tag in both the host tissue and the *in vitro* biomass is determined by hybridization to oligonucleotide arrays or PCR assay. Mutant strains that fail to proliferate *in vivo* but are capable of reproduction *in vitro* contain an inactivated gene essential for the colonization of specific niches. Recently Brown et al. (2000) used this method with the opportunistic fungal pathogen *Aspergillus fumigatus* and identified a gene (*paba*A) encoding para-aminobenzoic acid synthetase that was an essential factor in pulmonary aspergillosis. An increasing number of genes are expected to be identified and cloned from pathogenic fungi using this approach in the near future.

(c) Agrobacterium Tumefaciens Mediated Transformation (ATMT). *Agrobacterium tumefaciens*, the tumor-inducing bacterium has long been used for transformation and gene tagging of plants owing to its ability to transfer distinct parts of its Ti-plasmid DNA into the plant cells and integrate the plasmid DNA into plant chromosomes in a random fashion (Koncz et al. 1992). Transformation of yeast and a wide range of filamentous fungal species by *A. tumefaciens* has been reported recently (deGroot et al. 1998a,b; Piers et al. 1996). Mullins et al. (2001) determined the critical parameters affecting transformation efficiency and the copy number of T-DNA inserted into the genome of the wilt-causing fungus, *Fusarium oxysporum* using novel vectors. The authors proposed this method as an efficient alternative gene tagging tool in filamentous fungi.

2.2 Cloning by Biochemical Function

Detection of the biochemical activity of a protein may also create an opportunity to clone its encoding gene. This could most simply be achieved by purifying the protein of interest from complex protein mixtures. Alternatively, various heterologous expression systems that have been developed to detect either enzymatic activities or specific DNA-binding abilities of the gene products could be used.

2.2.1 Protein Purification

Continuously improving protein separation techniques provide more and more sophisticated tools for researchers aiming to clone genes encoding proteins with detectable biochemical activity. In this approach a crude protein extract is prepared from disrupted cells or from the extracellular environment of the target organism. The extract is then subjected to a number of purification steps needed to obtain a pure single protein. A partial amino acid sequence of the purified gene product is then determined and used to design degenerate oligonucleotide primers. Subsequently, a

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portion of the gene is amplified by PCR to generate a gene specific probe. Alternatively, antibodies raised against the purified gene products can be used to screen expression libraries.

2.2.2 Heterologous Expression

A yeast expression system using *S. cerevisiae* as heterologous host has been developed to clone fungal enzyme genes (Dalboge and Heldt-Hansen 1994). A cDNA library is constructed in an *Escherichia coli*–yeast shuttle vector containing an inducible yeast promoter from mRNA purified from fungal mycelium grown under conditions favoring enzyme production. Plasmid DNA isolated from pools of transformant *E. coli* is then used to transform yeast cells. Transformant yeast colonies are then replica-plated onto inducing the medium, containing substrates, which allow the detection of the enzyme of interest. This methodology requires that the fungal enzymes are translated, processed, and secreted in sufficient amounts and quality suitable for plate assays. The advantages of this method over traditional ones used for cloning enzyme genes are that it requires no preliminary information on the properties of the protein, needs no laborious protein purification procedures, and allows the cloning of genes whose products are synthesized in limited amounts by the source organism. Moreover, several different enzyme genes can be simultaneously cloned by replica-plating the yeast library onto media containing different substrates. Hundreds of enzyme genes including several enzyme families from a wide range of filamentous fungi have already been cloned using this strategy (Dalboge and Lange 1998).

Another cloning strategy based on yeast expression systems was developed to clone genes on the basis of specific DNA binding and transcription activating properties of their products (Saloheimo et al. 2000). The method seems to be of general applicability, however, an important requirement is that the yeast strain used as host should not express transcription factors that activate the promoter of interest. In this technique the target promoter is fused with a selectable yeast reporter gene (encoding the auxotrophic marker X), this plasmid is then introduced into the yeast host to give a reporter strain. A constitutively expressed cDNA library is prepared from the fungus in a yeast–*E. coli* shuttle vector containing another auxotrophic marker (Y). Subpools of the library are then used to transform the reporter strain and colonies that can grow on a medium lacking amendments for both the X and Y markers are isolated. Plasmids from these colonies that promote yeast growth in a medium lacking X, only in the presence of the reporter plasmid, contain transcription factors positively acting on promoter sequences introduced into the reporter strain. This method has been used to clone two transcriptional activator genes, *ace*1 and *ace*2 of the cellobiohydrolase gene, *cbh1* of *Trihoderma reesei* (Aro et al. 2001; Saloheimo et al. 2000).

2.3 Cloning by Sequence Similarity

A straightforward and technically simple methodology is to clone genes on the basis of their sequence similarities. This seems to be an attractive strategy especially when the target organism is difficult to culture or an efficient gene transfer system is lacking. It can be carried out simply by using a previously cloned gene as a heterologous probe in library screens to isolate its homologous sequence. However, the probability of success of the method can be severely limited due to insufficient homologies or the limited stretch of the homologous regions. Nevertheless, there are many examples in the literature reporting the successful application of heterologous gene probes to isolate their homologues from distantly related fungal species.

When sequence similarities between genes (or gene products) are confined to short regions, e.g., regions corresponding to specific functional domains, such as catalytic or substrate binding domains of enzyme families, alignment of these sequences gives information that can be used to design degenerate oligonucleotide primers. Using these primers in PCR reactions, portions of the genes of the targeted family can be amplified and then used as probes in library screening. With the growing accumulation of sequence data in gene banks, this approach is likely to gain broader application.

2.4 Cloning by the Transcriptional Regulation of the Gene

A number of approaches have been developed for cloning genes based on their regulation. Essentially, all these techniques involve the comparison of the transcribed (or translated) sequences present during different physiological, morphological, or developmental stages of an organism. Transcripts that appear exclusively, or more abundantly in a particular stage are assumed to originate from genes whose activity contributes to bring about or maintain the condition studied. In terms of experimental methodology, three main approaches, namely (a) hybridization-based techniques, (b) PCR-based techniques, and (c) protein-based techniques are used to detect differences in transcript abundance. However, a large variety of modifications of the basic protocols have recently been developed. The border line between the methods listed above is not always sharp, and, for example, some methods combine hybridization and PCR technology.

2.4.1 Hybridization-Based Techniques

Differential hybridization involves the preparation of a cDNA library from mRNAs obtained from cells grown under the conditions of interest. This is followed by a differential screening of replicas of this library by hybridization to separate first strand cDNA probes obtained from different conditions that are subjects of comparison. Clones that give

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unique signals, or significantly more intense signals, when hybridized to a specific probe, are then selected and analyzed further.

A more sophisticated version of the hybridization-based approach is subtractive hybridization, which involves the selective enrichment of specific sequences present in one sample but absent from another one. The population of cDNA molecules in the sample originating from the condition of interest (tester) is mixed with excessive amounts of nucleic acids from the other sample (driver), and these are then hybridized to each other. This is followed by physical separation of the single-stranded molecules. The single-stranded material contains sequences from genes with stage-specific expression properties different from the double-stranded nucleic acids that contain transcripts present in both samples (Duguid and Dinauer 1990; Hedrick et al. 1984).

In an advanced version of this method, physical separation is substituted by adaptor ligation and selective PCR amplification of transcripts originating only from the desired sample (Diatchenko et al. 1996).

2.4.2 PCR-Based Differentiation

Differential display of mRNA is a powerful method to identify and clone differentially transcribed genes (Liang and Pardee 1992). In general, mRNA populations of samples obtained from different conditions are reverse transcribed, and then PCR-amplification is performed with an arbitrary primer and an 'anchor' primer, homologous to the polyadenylated 3′ end of mRNA species but containing extra bases at the 3′ end allowing arbitrary selection of the template molecules. Amplification then generates specific fragments characteristic of the samples obtained from different conditions. These fragments can be separated by electrophoresis. Differentially expressed fragments, assumed to be portions of genes with stage-specific expression are then cloned and used as probes to screen gene libraries. A major advantage of this strategy over subtractive hybridization is that several differentially treated samples can be analyzed simultaneously.

2.4.3 Protein-Based Differentiation

Comparison of the abundance of gene products rather than transcripts in samples obtained from different conditions is another important approach for identifying and cloning differentially regulated genes. Furthermore, in specific cases this approach can offer a more relevant solution in circumstances when (a) protein abundance does not always correspond to mRNA abundance, (b) the samples to be compared may not always contain mRNA (e.g., when studying secreted gene products), and (c) the regulation of genes may occur at the post-translational level (proteolysis, glycosylation, etc.) (Pandey and Mann 2000). In general, protein extracts from different conditions are separated by two-dimensional gel electrophoresis. This involves a first-dimension isoelectric focusing of the proteins in a gel strip followed by separation in a second dimension according to the size of the polypeptides. Separated and fixed proteins are then transferred to a solid surface and stained resulting in condition specific patterns. These patterns can then be analyzed quantitatively with appropriate image analysis software. Molecules that show significantly different staining intensities or only occur in a specific condition denote polypeptides translated from differentially expressed messages or proteins subject to differential post-translational modification. These can be further analyzed by excision and the determination of partial amino acid sequence. The information obtained can be used to design oligonucleotide primers for the amplification of sequences that can be used as a gene-specific probe for screening libraries.

A good example of making use of this strategy in gene cloning from fungi was the isolation from *Penicillium decumbens* of the *epoA* gene encoding an epoxidase enzyme which plays a key role in the biosynthesis of fosfomycin, an antibiotic compound of commercial importance (Watanabe et al. 1999).

2.5 Cloning by Chromosomal Position

A number of cloning methodologies including map-based cloning and cloning by synteny exploit information on the position of a gene on a chromosome.

2.5.1 Map-Based Cloning

Map-based cloning (or positional cloning) involves the construction of a genetic map of the target organism and determination of the position of the target gene on the map. Molecular markers that co-segregate with highest frequency with the trait can then be used as probes to initiate chromosome walking in order to clone the genomic region containing the gene of interest. Since map-based cloning requires the identification of a detectable phenotype, this strategy could have also been included in Cloning by Biological Function as a cloning method based on biological function.

This approach is only applicable to fungal species that are amenable to classical genetic studies. The construction of a complete genetic map involves the assessment of the relative distances between phenotypic and molecular markers. This is achieved by calculating the frequency of recombination between the respective markers. Different types of molecular markers are used comprehensively to generate highly saturated maps. Restriction length polymorphisms (RFLPs) are detected by hybridizing randomly cloned DNA fragments to restriction digested DNA samples of fungal specimens. Randomly amplified polymorphic DNA fragments (RAPDs) are products of arbitrarily primed polymerase chain reactions. Amplified fragment length polymorphisms (AFLPs) are generated by ligating adaptors to restriction digested DNA fragments and selective amplification of a subset of the fragments (Vos et al. 1995). The generation of a fungal genetic map highly saturated with molecular markers

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increases chances of finding two closely linked markers on each side of the target gene. Such markers can then be used as probes in chromosome walking experiments to obtain overlapping clones containing the gene of interest. Once these clones are isolated the gene responsible for the trait studied can be identified by subcloning experiments followed by sequencing and/or mutant complementation.

2.5.2 Synteny

The co-linearity of genes, i.e., the conservation of gene orders among fungal genomes, can also be used in order to clone a gene. This strategy uses a conserved gene or a molecular marker found to be closely linked to the gene of interest in a model organism with a highly saturated genetic map. This gene or marker is then used as a probe in chromosome walking experiments on a library from the target organism. The success of this approach depends on the similarity of the genomes of the model and the target fungi. To date there has only been limited information published on the extent of synteny between fungal genomes. The existence of syntenic regions has been observed between *Magnaporthe grisea* and *Neurospora crassa* but no synteny was detected between *M. grisea* and the yeasts *S. cerevisiae* and *Candida albicans* (Hamer et al. 2001). As fungal genome projects proceed and, as a consequence, comparative genomics develops, the applicability and usefulness of this approach is likely to increase.

3 EXAMPLES OF GENE CLONING IN FUNGAL BIOTECHNOLOGY

Cloning of fungal genes has made a major contribution to a better understanding of the biochemical and cellular mechanisms that occur in the eukaryotic cell, it has helped to reveal the recognition process between cells of different origins and opened up new possibilities for improving productivity of fungi and plants. Some particular examples of these are highlighted below.

3.1 Towards the Understanding of the Reproduction Strategies in Fungi

A better knowledge on the reproduction strategies of fungi is needed in order to monitor changes in fungal populations or select the most appropriate disease control measures. In many fungi, the mating system is heterothallic, based on the interaction of two compatible strains of opposite mating type. Some species, however, are homothallic, and are capable of sexual reproduction within a single line. A third group of fungi, comprising thousands of morphological species, are considered as asexual taxa, that have either lost the ability to reproduce sexually or have a cryptic sexual cycle elicited by unusual environmental conditions or other unknown stimuli. Mating type is determined by *MAT* genes that encode transcriptional regulators, confer mating type identity, and control the development of fruiting bodies and meiospores. *MAT* genes have been cloned by using diverse strategies, including genomic subtraction, complementation of *MAT*-deletion mutants, and PCR-based techniques (Turgeon 1998). The latter proved to be especially useful for cloning mating type genes in a wide range of ascomycetes, as the *MAT* idiomorphs of these fungi contain two highly conserved sequences, called the α-domain and HMG-box sequences bound in *MAT-1* and *MAT-2* strains, respectively. These are easily amplified by using either group specific or degenerate primers (Arie et al. 1997). Cloning of *MAT* genes has provided a rapid and robust diagnostic procedure for mating type identification in the *Gibberella fujikuroi* species complex, including the determination of biological species and asexual lineages (Steenkamp et al. 2000).

3.2 Recognition Between Plants and Pathogenic Fungi

Cloned fungal genes helped to understand the recognition specificity between plants and pathogenic fungi and reveal the molecular background of the gene-for-gene relationship, a theory first proposed more than 40 years ago (Flor 1955). The tomato–*Cladosporium* interaction has been widely used as a model system in these investigations.

Cladosporium fulvum (now correctly named *Mycovellosiella fulva*), a biotrophic fungal pathogen causes leaf mold on tomato. Plant breeders have introduced several resistance genes (called *Cf* genes) against this pathogen from wild *Lycopersicon* species into commercial tomato cultivars. As a result, many races have emerged in *C. fulvum* that are able to overcome the *Cf* genes built in tomato in various combinations. A typical gene-for-gene relationship has been seen in the tomato–*C. fulvum* system, in which each plant resistance gene (*Cf*) has its avirulence gene (*Avr*) counterpart in the fungal population. Interaction between tomato cultivars and *C. fulvum* races that contain properly matching *Cf* and *Avr* genes, respectively, results in recognition, leading to a hypersensitive defense reaction (HR) by the plant. If partners containing nonmatching *Cf* and *Avr* genes meet, recognition fails to occur and the fungus successfully invades the plant tissues.

Experiments on the tomato–*Cladosporium* system resulted in the cloning of the first fungal avirulence gene. A race-specific protein was first isolated from the intercellular fluids of tomato leaves infected with *C. fulvum* race 9. This protein proved to be an elicitor, capable of inducing HR on tomato cultivars that carry the *Cf9* resistance gene. The cDNA of this protein was identified and the corresponding avirulence gene, designated *Avr9* was isolated from the fungus (Van Kan et al. 1991). Race 9 strains of *C. fulvum* were unable to cause disease on cultivars harboring the *Cf9* gene, as these plants recognized their AVR9 protein and produced HR. The crucial role of *Avr9* was proven in subsequent

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experiments where *C. fulvum* races, virulent on *Cf9*-tomato plants, were transformed with the *Avr9* gene. The transformants were avirulent and the plants showed HR. In contrast, the disruption of *Avr9* in race 9 strains gave virulent mutants (Marmeisse et al. 1993; Van der Ackerveken et al. 1992).

AVR9 is a cysteine-rich hydrophobin protein. Hydrophobins confer hydrostatic properties on the surfaces of dry-spored fungi, assist in hyphal binding and appressorium development, and are essential fungal compounds. As hydrophobins are located on the outermost parts of fungi, they may be among the first fungal components recognized by host cells, and so may be particularly suited to eliciting plant responses.

Similar cysteine-rich elicitors have been identified in other races of *C. fulvum* and one of these, encoded by *Avr4*, causes HR on tomato cultivars containing the *Cf4* resistance gene. Surprisingly, when a 510-bp segment of *Avr4* was used as a probe, *Avr4* homologues were identified in seven additional races of *C. fulvum* not recognized by *Cf4*-tomato plants. The RFLP-analyses revealed no differences among the homologues, and this finding seemed to contradict the avirulence gene theory. However, when the *avr4* homologues were subjected to sequence analysis, most *avr4* homologues were found to contain a single base pair substitution in one of their cysteine codons and in all cases, the cysteine codon (TGT) was replaced by to a tyrosine one (TAT) (Joosten et al. 1994). As cysteine plays an important role in the formation of the tertiary structure of proteins, point mutations in these hydrophobin coding genes would result in structurally modified AVR proteins and the pathogen could thus avoid recognition by plant receptors coded for by the corresponding *Cf* resistance gene. More recent studies have demonstrated that besides the cysteine–tyrosine substitutions, other amino acid changes in the *avr4* gene products may also contribute to the elicitor variability and alter virulence in *C. fulvum* (Joosten et al. 1997).

Identification of the *Avr* genes has stimulated studies on similar genes of other fungal pathogens (Laugé and de Wit 1998). Knowledge of the *Avr* genes cloned from *C. fulvum* not only helped to give a molecular explanation for the gene-for-gene relationship, but could also be utilized in developing a novel molecular resistance breeding approach. This strategy is based on the simultaneous expression of a *Cf* gene and its corresponding *Avr* gene in the same plant. If cassettes containing a matching pair of these genes are prepared and placed under the transcriptional control of a pathogen inducible promoter, attacks of micro-organisms others than *C. fulvum* are recognized by this promoter and the *Cf–Avr* construct initiates a rapid, efficient, but localized defense response (deWit 1992; Joosten and deWit 1999).

Fungal genes could also be used to improve plant resistance against bacteria and fungi either by enhancing or supplementing the existing plant defense arsenal. The plant–pathogen interaction triggers a cascade of defense steps, including an oxidative burst, i.e., a rapid and transient production of active oxygen species (superoxide anion radical, hydroxyl radical, and hydrogen peroxide), followed by cell-wall reinforcements, phytoalexin synthesis, and accumulation of pathogenesis-related proteins. Enhanced H_2O_2-generation has been shown to be one of the means by which plant resistance could be improved by using a gene of fungal origin (Wu et al. 1995). H_2O_2 supports plant defense by not only inhibiting or killing the pathogens, but acts also as a secondary signaling molecule that activates the enzymes involved in cell-wall reinforcement and phytoalexin biosynthesis. A glucose oxidase gene, *GO*, has been cloned from *Aspergillus niger* and used to transform potato. Both leaves and tubers of the transgenic plants were found to accumulate high levels of glucose oxidase and the majority of the recombinant enzyme was secreted into the apoplast. As glucose oxidase catalyzes the generation of H_2O_2 from glucose, transgenic tubers were found to show strong H_2O_2-mediated resistance against the soft rot pathogen, *Erwinia carotovora*. Furthermore, transgenic leaves expressing the *GO* gene also exhibited increased H_2O_2-levels and the elevated H_2O_2-production resulted in significantly reduced numbers and sizes of leaf lesions caused by the late blight fungus, *Phytophthora infestans*.

Certain classes of pathogenesis-related (PR) proteins, induced in plants by biotic or abiotic stress factors, have chitinase and glucanase activities with a direct antifungal effect. They are therefore regarded as important enzymes of plant defense mechanism. Chitinase genes of plant origin have been used to improve plant resistance via genetic engineering, but the level of resistance obtained in this way generally proved to be insufficient. The main reason for this is the limited antifungal activity of plant chitinases. Chitin degrading enzymes of antagonistic fungi are about two magnitudes more efficient in their antifungal activity than the corresponding plant enzymes; their use in the production of resistant transgenic plants would appear to be more promising. Lorito et al. (1998) successfully improved plant resistance by means of the 42 kDa endochitinase gene (*ech42*), cloned from the biocontrol fungus *Trichoderma harzianum*. Both the genomic copy and the cDNA of this gene, driven by a 35S promoter, were introduced into tobacco and potato. The levels of heterologous chitinase activity varied greatly among the transgenic plants, but selected lines that showed high levels of *ech42* expression proved to be completely resistant to a wide range of fungi, including *Alternaria alternata*, *Botrytis cinerea*, and *Rhizoctonia solani*. The 42-kDa *Trichoderma* endochitinase represented 0.01–0.5% of the total protein content of the transgenic plants, and the physico-chemical properties of the heterologous enzyme were similar to those of the purified fungal enzyme. It is worth mentioning that both the transcript and its protein product were processed correctly by the plants. The high levels of resistance observed in several lines of these transgenic plants can be explained by the strong direct antifungal activity of the *Trichoderma* endochitinase, the appropriate location of the enzyme (it was found to accumulate in the extracellular space) and its ability to split chitin oligomers from the invading fungi, that in turn served

as elicitors, inducing additional plant defense steps that acted synergistically with chitinase.

3.3 Cloning and Utilization of Genes Encoding Metabolic Pathways

β-Lactams, including penicillins and cephalosporins, are still the most important of the large variety of antibiotics secreted by fungi. These compounds are synthesized by condensation of L-α-aminoadipic acid, L-cystein, and L-valine to form δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), which is further converted to isopenicillin N (IPN). Subsequent steps include acetylation or epimerization yielding penicillin G (produced by *Penicillium chrysogenum*) and penicillin N (produced mainly by *Acremonium chrysogenum*), respectively. Penicillin N is then converted to cephalosporin by a bifunctional expandase-hydroxylase and an acetyltransferase. Almost all genes involved in β-lactam biosynthesis have been cloned (Alvarez et al. 1993; Díez et al. 1990; Gutiérrez et al. 1992; Samson et al. 1985;1987). The genes *pcbAB* and *pcbC*, which control ACV and IPN synthesis are common in all β-lactam producing fungi; *penDE* coding for an IPN acetyltransferase, the first specific enzyme of the penicillin G biosynthetic pathway is present exclusively in *P. chrysogenum*, whereas *A. chrysogenum* harbors a *cefEF–cefG* cluster, that includes the genes that code for the later steps of cephalosporin synthesis. Cloned β-lactam biosynthesis genes have been utilized to engineer overproducing strains by overexpressing either the *cefG* gene in *A. chrysogenum* (Gutiérrez et al. 1997) or the *acvA* gene (a homologue of *pcbAB*) in *A. nidulans* (Kennedy and Turner 1996). An additional gene, named *cahB*, and that not linked to the *cef* cluster, has also been found to affect cephalosporin C production in *A. chrysogenum*. This gene encodes for cephalosporin acetylhydrolase, an enzyme converting cephalosporin C to an inefficient side-product, deacetylcephalosporin C. Inactivation of *cahB* was suggested as a mechanism by Velasco et al. (2001) to obtain strains that accumulate higher amounts of intact cephalosporin C without losses caused by deacetylcephalosporin C conversion.

Gibberellins are another important group of secondary metabolites synthesized by fungi. These tetracyclic diterpenoid plant growth regulators are produced by *Gibberella fujikuroi*, the causal agent of the 'bakanae' disease of rice. Commercially available gibberellin preparations are widely used for stimulating the growth of rice seedlings and enhancing the germination of barley seeds used for malt production. Improved strains, obtained by traditional mutagenesis are available for industrial production of gibberellins, but further enhancement of these strains by using recombinant DNA methodologies would be of great interest. Efforts to understand the molecular background of gibberellin biosynthesis have resulted in the cloning of the *cps* gene, that codes for copalyl diphosphate synthase (CPS) (Tudzynski et al. 1998). The CPS catalyzes the first specific step of gibberellin synthesis by branching off from the general isoprenoid pathway and producing isokaurenes, the precursor compounds of gibberellic acids. The expression of this gene is strongly repressed by high concentrations of ammonium, and expression increases considerably in late stationary growth. The directed up-regulation of the copalyl diphosphate synthase gene could be one of the means to increase gibberellin production during fermentation.

Ergot alkaloids produced by members of the *Clavicipitaceae* are also an economically important group of fungal secondary metabolites. These compounds are widely used in human therapy to aid childbirth or ease migraines. Alkaloid extracts are obtained from sclerotic or mycelial mats of the *Sphacelia* anamorphs cultured in large-scale fermentors, but these extracts contain undesirable compounds, and so cloned genes of the ergot biosynthesis pathway are needed to engineer strains which produce more specific and safe ergot compounds with less impurities. Several members of a gene cluster involved in ergot biosynthesis have been recently identified including *cpd1*, which encodes dimethylallyltryptophan synthase, the first specific enzyme of the ergot pathway, as well as a number of peptide synthetase and oxidase genes (*cpps1*, *cpps2*, *cpps3*, *cpox1*, *cpox2*, *cpox3*) that control later steps of the pathway. Site-directed mutagenesis of one or more *cpps* genes, as suggested by Tudzynski et al. (2001) would be an elegant and promising approach to produce strains secreting new or more efficient ergot compounds.

3.4 Genes of Potential Uses in Yeast Improvement

Although there is a strong public aversion to use of genetically engineered yeast strains in brewing, wine-making, or baking, the recombinant DNA technology suitable for producing improved strains is easily accessible. A number of genes, that have been cloned recently could be utilized in strain-breeding programs. The major targets for manipulation in *S. cerevisiae* strains are improved flocculation, elimination of undesired by-products, extension of substrate utilization, increased ethanol and organic acid tolerance, as well as enhanced tolerance against osmotic stress. Genes controlling these traits are therefore of commercial interest.

Yeast flocculation is due to a reversible binding of cell-surface lectins and mannans and results in the aggregation of cells into flocs that can easily be removed by sedimentation. The efficient and appropriately timed removal of yeast cells by the end of the fermentation process is an important step in obtaining clear, well-flavored beers, but the prerequisite of sedimentation is a good flocculation. Several flocculation specific genes have been isolated, and one of them, *FLO1*, has been used (Kobayashi et al. 1998) to improve the timing of flocculation. The *FLO1* promoter was replaced by the promoter of the heat-shock protein gene, *HSP30* (Verstrepen et al. 2000), which is induced by sugar depletion and high ethanol concentration, factors prevailing at the end of the brewing process. Optimal flocculation was obtained with

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engineered strains harboring the *HSP30-FLO1* fusion construct, and so allowed for a properly timed sedimentation under laboratory conditions.

Glycerol and isoamyl acetate are important flavoring components of beverages, and there are therefore strain improvement programs that aim to increase the yields of these compounds during fermentation. Genes that encode for the key enzymes of glycerol and isoamyl acetate synthesis could be used for engineering strains that could overproduce these compounds. *GPD1*, encoding glycerol-3-phosphate dehydrogenase has been successfully used to enhance glycerol production of wine yeast strains (Michnick et al. 1997). The *AFT1* gene, encoding an alcohol transferase which catalyzes the formation of ester bonds between acetyl CoA and alcohols, is another target of strain improvement programs. Overexpression of *AFT1* resulted in a substantial increase in isoamyl-acetate production in both sake and wine yeast strains (Fuji et al. 1994; Lilly et al. 2000).

Commercial preparations of glucoamylases are widely used as additives to degrade dextrins for producing light lager-type beers. However, this supplementary enzymatic treatment makes the fermentation process more complicated and hence, more expensive. An alternative solution of the problem is the construction of engineered yeast strains capable of dextrin degradation. The amyloglucosidase encoding *STA2* gene, cloned from *Saccharomyces diastaticus* was successfully used to produce a recombinant lager-brewing yeast strain, which secreted the enzyme and showed improved carbohydrate degradation capabilities (Perry and Meaden 1988).

The addition of various polysaccharide-degrading enzymes, including pectinases, glucanases, xylanases to grape fruit prior to fermentation improves the flavor and color of the wine. Several attempts have been made to replace the use of enzyme preparations by developing recombinant yeast strains capable of secreting these enzymes. One of the most successful approaches to this was the work of Gonzales-Candelas et al. (1995), who used the pectate lyase gene of *Fusarium solani* to transform wine yeast and so produced transformants with a polypectate degrading capability.

Molasses is a favored substrate for mass production of baker's yeast. This material contains large amounts of raffinose, a trisaccharide (fructose + galactose + glucose), which is hydrolyzed by the yeast to fructose and melibiose (galactose + glucose). Melibiose is not utilized further, as baker's yeast strains lack the melibiase (α-galactosidase) enzyme. The molasses utilizing capacity of baker's yeast has been successfully improved by transferring the melibiase encoding gene, *MEL1*, from a brewers' yeast strain into the baking strain obtaining thus transformants which produced substantial amounts of α-galactosidase (Liljeström-Souminen et al. 1988).

S. cerevisiae can also be used for industrial ethanol production by fermenting sugar components of lignocellulose hydrolysates. However, these hydrolysates contain phenolic compounds that are strong inhibitors of the fermentation process and yeast growth. An enzymatic detoxification method, based on a laccase extract prepared from the lignin-degrading basidiomycete *Trametes versicolor* has been developed for removing phenolic compounds prior to fermentation. The use of genetically modified yeast strains with phenol degrading capability promises a single-step alternative for this expensive and laborious enzymatic procedure. Two laccase genes (*lac1*, *lac2*) cloned from *T. versicolor* have been used to transform two *S. cerevisiae* strains (Cassland and Jönsson 1999). Stable transformants with the ability to secrete heterologous laccase were obtained only from one that had been transformed by *lac2*, indicating that both the recipient strain and the laccase gene should be carefully chosen for the strain-improvement program. The fermentation temperature was also found to be an important factor, and the positive transformants exhibited 16-times higher laccase activity when cultured at 19°C instead of 28°C.

3.5 Cloned Fungal Sequences and Improved Heterologous Protein Production

Enzymes are increasingly being used to replace environmentally less-friendly chemical technologies in the paper, textile, fodder, or detergent industries. The majority of these enzyme preparations are produced as heterologous proteins secreted by fungi, including *Aspergillus*, *Fusarium*, *Kluyveromyces*, and *Trichoderma*. In order to be economically viable, enzymes obtained from fungi should be as cheap as possible, but this requirement can only be met by constructing high production strains for industrial use. Although fungi used for heterologous enzyme production secrete exceptionally large amounts of proteins into the growth medium, this secretion capacity still needs improvement. The major limiting factors of heterologous protein secretion in fungi are insufficiencies during the post-translational modification process of these proteins. The genetic regulation of protein folding, glycosylation, and the whole secretory machinery are therefore subjects of increasing interest. Studies on these mechanisms have resulted in cloning sequences that are promising targets for the improvement of heterologous enzyme production in fungi.

Chaperones and foldases are enzymes that assist in protein secretion and have been collectively termed helper proteins. A number of such enzymes are already known from fungi and the corresponding genes have also been cloned and characterized. Information is available for the successful manipulation of some helper protein encoding genes. For example, overexpression of *KAR2*, a protein-binding chaperone gene, resulted in a 20-fold higher bovine prochymosin production in yeast (Harmsen et al. 1996). Similarly, increased PDGF (human platelet-derived growth factor) production was obtained in *S. cerevisiae* by overexpressing the foldase encoding the *PDI* gene (Robinson et al. 1994). However, in other experiments the genetic manipulation of these helper protein systems has given contradictory results, and this may be explained by the

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complexity of the molecular interactions involved in the secretion pathway. Conesa et al. (2001) concluded that the function of helper proteins depends on their targets and this may result in opposite effects on the secretion of the same heterologous protein.

3.6 Fungal Genes for Bio-Remediation Processes

One of the adverse effects of industrialization is the increased release of pollutants into the environment. Heavy metals, including arsenic, cadmium, copper, iron, lead, and mercury are among the most dangerous pollutants on abandoned mining or industrial areas. Plants are especially suitable for removing heavy metals from polluted soils, as they can accumulate elements in higher amounts than needed.

The efficiency of phytoremediation could be improved by producing genetically engineered plants, capable of increased metal uptake that could accumulate exceptionally high levels of heavy metals in their tissues or may transform and volatilize the pollutants (Krämer and Chardonnens 2001). Heavy metal tolerance in oil seed rape (*Brassica oleracea*) has been improved by transformation, with the methallothionein gene, *CUP1*, cloned from *S. cerevisiae*. Transformants were able to grow in a hydroponic medium, containing 400 μM cadmium, whereas control plants could tolerate only 25 μM Cd^{++} under the same culture conditions. Furthermore, *B. oleracea* plants harboring the *CUP1* gene accumulated 10–70% higher concentrations of cadmium in their leaves than the wild-type plants when both were grown in a medium containing 25 μM cadmium (Hasegawa et al. 1997). Iron tolerance in tobacco has been substantially enhanced by the introduction of two ferric reductase encoding genes (*FRE1*, *FRE2*) from yeast. The iron content measured in the leaves of transformants, grown in liquid culture medium was 50% higher, than that measured in the leaves of control, nonengineered plants (Samuelson et al. 1998).

3.7 Use of Cloned Genes in Mushroom Improvement

More than 5 million metric tons of cultivated mushroom are produced annually worldwide, and there is an increasing demand for new varieties with more efficient substrate utilization, improved quality, and tolerance to mechanical injuries encountered during harvest and transport. The efficiency of breeding edible mushrooms based on traditional techniques is limited by factors like sexual incompatibility, which largely prevents out-crossings, and or the lack of homokaryotic spores in many species. These limitations make it very difficult to obtain near-isogenic parental lines. Molecular breeding strategies utilizing cloned genes provide potential for the production of transgenic mushroom varieties with improved traits, including productivity, disease resistance, or marketability.

Genes involved in substrate utilization are the most promising targets to clone and use in strain improvement. Edible mushrooms are mostly produced on commercial composts containing lignocellulose as the major organic component. Although all the main groups of cultivated mushrooms, including *Agaricus*, *Flammulina*, *Lentinulla*, and *Pleurotus* spp. have complex lignocellulose degrading enzyme systems comprised of laccases, peroxidases, glucanases, xylanases, and cellobiohydrolases, their substrate utilization efficiency could be improved through broadening the existing spectrum of enzymes by the introduction of various peroxidase and/or hydrolase encoding genes from heterologous sources. A number of genes, encoding various lignocellulose degrading enzymes have been cloned from *Agaricus bisporus*, including two laccase genes (*LCC1* and *LCC2*) as well as a cellulase (*CEL1*), a cellobiohydrolase (*CEL2*), an endoxylanase (*XLNA*), and a mannosidase (*CEL4*) gene (Chow et al. 1994; deGroot et al. 1998a,b; Raguz et al. 1992; Yagüe et al. 1997). As transformation techniques used for genetic manipulation of cultivated mushrooms have improved in recent years (Stoop and Mooibroek 1999), constructing transgenic mushrooms capable of more efficient substrate utilization seems technically possible.

Another target of mushroom breeding is to improve the marketability of the product. Fresh mushrooms may suffer from brown discoloration caused by mechanical injury, bacterial infection, or natural senescence. Polyphenoloxidases, most notably tyrosinases are responsible for this discoloration. These enzymes catalyze the conversion of tyrosine to *o*-diphenol-3,4-dihydroxyphenylalanine and then to dopaquinone, which is further converted to melanin and melanochrome, two major pigments found at high concentrations in browned fruiting bodies. Two tyrosinase encoding genes, *AbTYR1* and *AbTYR2* have been cloned from *A. bisporus* and used in antisense orientation to obtain stable transformants with silenced activity of the native tyrosinase genes (Stoop and Mooibroek 1999). However, the highly complex nature of melanin biosynthesis allows switching on alternative pathways, and therefore silencing these two tyrosinase genes may not completely prevent discoloration. The antisense strategy could be one of the most rational ways of delaying discoloration.

A third important area of mushroom breeding is to reduce the level of sporulation in certain species, as spores of some species like the oyster mushroom (*Pleurotus ostreatus*), can pose serious allergic hazards to mushroom workers. Nonsporulating oyster cultivars may be produced in the near future by manipulating the *PoDMC1* gene, which has recently been cloned from *P. ostreatus* (Mikosch et al. 2001). This gene, a homologue of the yeast *DMC1* gene (known to be essential for meiospore production), is expressed only in spores and sporogenic cells of *P. ostreatus*, but not in vegetative cells; its targeted disruption could therefore result in the production of sporeless mutants.

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3.8 Fungal Genes with Human Relations

Alkaptonuria, a hereditary human disorder, results in serious arthritis as a consequence of a dysfunction in the phenylalanine–tyrosine degradation pathway. Decomposition of phenylalanine to fumarate + aceto-acetate requires the activity of six enzymes, including homogentisate dioxygenase. A deficiency of this enzyme leads to the accumulation of homogentisate metabolites in the connective tissues resulting in a degenerative arthritis. The hereditary nature of this disorder has long been known. The biochemical background of the disease was determined in the late 1950s, but cloning of the "alkaptonuria" gene was considered unlikely due to the high complexity of the human genome.

A fungal model has been used to solve the problem. As filamentous fungi and humans use the same phenylalanine–tyrosine degradation pathway, investigations were initiated to isolate the fungal homologue of the "alkaptonuria" gene, encoding homogentisate dioxygenase. *A. niger* mutants unable to degrade phenylalanine were selected by the accumulation of a typical alkaptonuric pigmentation, when grown on phenylalanine containing medium. As a result a cDNA clone of the *hmgA* gene was successfully isolated. The HmgA protein produced in *E. coli* showed homogentisate dioxygenase activity (Fernández-Cañon and Peñalva 1995). Database searches based on this information led to the identification of the human homogentisate gene, *HGO*, which consists of 14 exons and is 53,563 bp in length. A number of pathogenic mutations have subsequently been identified in alkaptonuria-affected families, most of them represented missense mutations (Rodriguez et al. 2000). Based on these studies, implementation of genetic screens for this heritable metabolic disorder can now be developed.

The successful results achieved in alkaptonuria research have encouraged further studies with the fungal models to determine the molecular bases of other human hereditary metabolic disorders including phenylketonuria, tyrosinemia, and molybdenum cofactor deficiency (Peñalva 2001).

Investigations of organization, function, and regulation of certain fungal genes may further allow a better insight into the aging process of cells and organisms, and may therefore be helpful in understanding the genetic control of human age-associated disorders. There are striking similarities between fungal and mammalian aging. Fungal cells, like many mammalian and human cells, have a restricted number of divisions when cultured even under optimal conditions. Recently, several genes that affect aging have been cloned from *S. cerevisiae* (Sinclair et al. 1998). Two *ras* homologues, functionally interchangeable with mammalian *ras* genes have been cloned from yeast and one of them, *RAS2* was found to extend the life span of yeast clones when overexpressed (RAS proteins are key elements of intracellular signal transduction). Two other genes, *LAG1* and *LAG2* (LAG = longevity assurance gene) that encode for putative transmembrane proteins and are preferentially expressed in young cells of *S. cerevisiae*, also exerted a definitive influence on life span. Deletion of these genes reduced the mean life span by some 50–60%, whereas their overexpression showed an opposite effect. A yeast homologue (*sgs1*) of the human WRN gene (responsible for a human progeroid disorder, called Werner's syndrome) encoding a helicase has also been cloned and studied in detail. Normally, the SGS1 protein accumulates in the nucleolus, predisposing it to DNA damage, thus causing genomic instability which leads to a strongly reduced life span with typical senescence symptoms like sterility or fragmentation of the nucleolus (Sinclair et al. 1997). All these findings will further encourage work with simple yeast model in order to understand universal elements of cellular aging that may be common in fungi and humans.

4 CONCLUSIONS

In this chapter the strategies used in cloning genes from fungi are discussed and illustrated, with selected examples the application of gene cloning in several important areas of fungal biotechnology. Cloning genes from fungi used as model organisms has proven to be an important experimental tool that has provided valuable information for understanding basic biochemical and cellular processes of the eukaryotic cell. Gene cloning methodologies have also been utilized to provide useful genes for application in various biotechnological processes.

In the near future the number of fungi with fully sequenced genomes is likely to increase. The information from these genomes will show the structure of the genetic material, but important questions such as the identification of functional units, i.e., genes and the assignment of biological and/or biochemical functions to these genes, still remain to be solved. Some of the gene cloning techniques outlined above will certainly play an important role in the functional characterization of the ORFs identified in model organisms. Furthermore, gene cloning techniques remain valuable experimental tools in studying the vast majority of fungi that are not subjects of genome sequencing programs. Many of these organisms contain genes that control cellular processes affecting traits of significant values in terms of industrial, agricultural, or medical biotechnology. Gene cloning methods will continue to be exploited in the identification of the key genetic components of processes of potential biotechnological significance.

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9

Transformation and Gene Manipulation in Filamentous Fungi: An Overview

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1 INTRODUCTION

The development of suitable gene transfer systems is a major prerequisite for molecular genetic and biochemical investigations in various organisms, including the filamentous fungi. Molecular transformation involves two main components: (a) an appropriate vector containing a selectable marker and (b) a suitable transformation procedure for the introduction of the corresponding vector into the respective fungal system. Finally a sustainable replication of the transforming DNA has to be achieved either by integration of the vector into the genome or by applying an autonomously replicating vector system.

Over the last three decades there has been substantial progress in developing transformation techniques for filamentous fungi. Most research has concentrated on unicellular yeast *Saccharomyces cerevisiae* and the two main model organisms for filamentous fungi, *Neurospora crassa* and *Aspergillus nidulans*. Nevertheless, transformation has also been successfully applied to a number of other filamentous fungi as reported in a number of excellent reviews and book chapters that also cover expression systems for specific groups of fungi [e.g., Ballance (1991); Bodie et al. (1994); Fincham (1989); Frommer and Ninnemann 1995; Goosen et al. (1992); Mach et al. (1994); May (1992); Radzio and Kuck (1997); Riach and Kinghorn (1996); Ruiz-Diez (2002); Timberlake and Marshall (1989); Turner (1994); van den Hondel and Punt (1991); Verdoes et al. (1995)]. The above mentioned development of transformation systems for the two filamentous model organisms (*N. crassa* and *A. nidulans*) has provided a basis for transferring such techniques to less investigated but economically and industrially important fungal species (Timberlake and Marshall 1989).

The first fungal transformation was reported by Mishra and Tatum (1973), who described the conversion of auxotrophic *N. crassa* mutants (inositol requiring) to prototrophy at very low rates by exposing them to total wild type DNA and calcium chloride. A few years later, Hinnen et al. (1978) obtained significantly increased competence of DNA uptake by removing cell walls, thereby creating protoplasts, and they became the first researchers to transform a *S. cerevisiae leu2*- mutant. The protoplast technique, which was already proven to be appropriate for yeast transformation, was applied to *N. crassa* (Case 1986; Case et al. 1979) and *A. nidulans* (Tilburne et al. 1983). These major breakthroughs in fungal transformation were subsequently utilized with a variety of other fungal species.

2 TRANSFORMATION PROCEDURES FOR FILAMENTOUS FUNGI

Various techniques have been used to introduce DNA into the nuclei of filamentous fungi, these include protoplasting, chemical treatments, application of electric pulses, or physical damage. To date protoplasts are most frequently used for such insertions of exogenous DNA. In addition to the generally used calcium-chloride/PEG method, a liposome- (Radford et al. 1981) and an electroporation-based method for protoplasts have also been described (Goldman et al. 1990; Thomas and Kenerley 1989). There are also some methods that use intact cells for transformation, including exposure of cells to lithium acetate and transforming DNA (Dhawale et al. 1984), effectual mixing of cells in the presence of glass beads and DNA (Costanzo and Fox 1988), and more recently

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particle bombardment (Klein et al. 1987), and *Agrobacterium tumefaciens*-mediated transformation (Bevan 1984).

2.1 Preparation of Protoplasts and Regeneration

Protoplasts are obtained by incubating mycelia or spores with different cell-wall degrading enzymes under osmotically stabilized conditions. Early protocols involved enzymatic degradation with a variety of enzyme cocktails obtained from different filamentous fungi (Peberdy 1985). The efficiency of the cell wall degradation depends on the wall structure of the particular fungal species. Commercially available mixtures were employed in protoplasting procedures in order to avoid the laborious preparation of such enzyme cocktails. Several enzyme mixtures have been used including enzymes isolated from snail stomach, (e.g., Helicase and Glusulase), and an enzyme concentrate from *Arthrobacter luteus* (Zymolase 100T), although the most frequently (Novozyme 234) enzyme cocktail is derived from *Trichoderma viride* (Riach and Kinghorn 1996). All such preparations contain a complex mixture of hydrolytic enzymes, consisting mainly of 1,3 glucanases and chitinases. A comprehensive review of different procedures and enzymes used for protoplasting has been produced by Peberdy (1989).

Protoplasts have to be osmotically stabilized throughout their preparation by sodium chloride, magnesium sulphate, manitol, or most frequently by sucrose or sorbitol. Concentrations between 0.8 and 1.2 M function as good osmotic stabilizers (May 1992) and lead to regeneration rates of up to 90%. In several protocols [e.g., Penttila et al. (1987)], storage of protoplasts at -70 to -80°C has been reported, this is a time saving procedure, but in several cases storage has been accompanied by a drastic reduction of transformation frequency. Irrespective of the transformation technique, transformed protoplasts are plated in an appropriate osmotically stabilized overlay regeneration medium, containing either a toxicant or another selective agent. For special applications, selection pressure is applied later through a second overlay step.

2.2 Polyethylene Glycol-Mediated Transformation

The polyethylene glycol (PEG)/calcium chloride method is the most frequently used transformation technique for filamentous fungi. The established protocols use protoplasts at a concentration of 5×10^7–5×10^8, and these are treated with a mixture of PEG, calcium chloride, and transforming DNA, such as described by Balance et al. (1983) and Tilburne et al. (1983) for *A. nidulans*. The DNA molecules are apparently internalized during the PEG induced protoplast fusion, whereas no transformation occurs when PEG is omitted (Timberlake and Marshall 1989). The PEG is typically used at a molecular weight of 4000; and in some published methods calcium chloride has been replaced by other alkali cations such as lithium chloride (Ito et al. 1983).

2.3 Liposome-Mediated Transformation

Liposome-mediated transformation has been reported once for *N. crassa* (Radford et al. 1981). Due to difficulties in the reproducibility of the preparation of liposomes and the positive results obtained from other procedures this method has not been applied further.

2.4 Electroporation

Electroporation involves the electrical permeabilisation of biomembranes and this has been reported as a relatively simple physical transformation method for both pro- and eukaryotic cells (Förster and Neumann 1989; Potter et al. 1984; Riggs and Bates 1986). In filamentous fungi, such a permeabilisation technique has been used with protoplasts, conidia, germinated conidia, and hyphal fragments that were treated with a short duration high amplitude electric field, and this has been described for several species including *Colletotrichum*, *Neurospora*, *Aspergillus*, *Beauveria*, *Penicillium*, *Leptosphaeria*, *Fusarium*, *Ustilago*, and *Trichoderma* (Bakkeren and Kronstad 1993; Chakraborty et al. 1991; Goldman et al. 1990; Redman and Rodriguez 1994; Ward et al. 1989). In most of these protocols, "competent" cells are prepared by simple techniques as opposed to the more complex purification steps needed for standard protoplast-based methods. The main advantages of electroporation are the simplicity of preparing sensitive cells and a widely documented increase in transformation reproducibility. In some cases a higher mitotic stability of the transformants has been reported [e.g., Goldman et al. (1990)].

Although protoplast-dependent transformation techniques have proved successful, they do have some major drawbacks: (a) One major disadvantage is the variability of the efficiency of protoplast formation and regeneration for different strains, (b) for several fungal strains, protoplasts have to be prepared freshly for each transformation experiment, and (c) protoplasts often contain more than one nucleus, and this can result in a long and time-consuming purification of transformants to obtain homokaryons.

2.5 Lithium Acetate-Based Transformation

Several attempts have been investigated to overcome the above mentioned problems. An early alternative to protoplast-based transformation was the development of the lithium acetate method initially described for *S. cerevisiae* (Ito et al. 1983), where the exposure of fungal cells to 0.1 M lithium acetate and DNA gave encouraging results. Subsequently, a similar procedure has been successfully applied to *N. crassa* (Dhawale et al. 1984) and *Coprinus cinereus* (Binninger et al.

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1987) by treating germinating spores with 0.1 M lithium acetate to give an alkaline metal-based increase in permeability for the transformation. Nevertheless, the mechanism by which alkali metals assist uptake of the transforming DNA is not fully understood. This technique has not been widely used for transformation of filamentous fungi as the reported transformation frequencies are very low compared to those obtained from protoplast-based methods [e.g., Dhawale et al. (1984)].

2.6 Biolistic Transformation

There has been one report of transformation brought about by physical damage by vigorously mixing cells with transforming DNA and glass beads (Costanzo and Fox 1988). Similar promising methods of bombarding cells with particles functioning as carriers for transforming DNA have been applied to several fungal species including *S. cerevisiae*, *A. nidulans*, *Paecilomyces fumosoroseus*, *Trichoderma harzianum*, *T. reesei*, *Gliocladium virens*, *Mucor circinelloides*, *N. crassa*, and *Magnaporthe grisea* (Aramayo et al. 1989; Barreto et al. 1997; Gomes-Barcellos et al. 1998; Hazell et al. 2000; Herzog et al. 1996; Klein et al. 1992; Lorito et al. 1993; Riach and Kinghorn 1996). In case of filamentous fungi, particles are frequently delivered to conidia placed on agar plates.

Particle bombardment was described for the first time by Klein et al. (1987). It employs high-velocity microprojectiles to deliver nucleic acids into intact living cells and tissues. The procedure involves coating the DNA on small diameter microbeads (0.1–30 μm) of tungsten, gold or glass. Lambda phage or dried *Escherichia coli* cells either coated with or containing the respective transforming DNA have also been used successfully. The bombardment is generally undertaken in a vacuum chamber in order to lessen the effect of air on the velocity of microprojectiles. When originally developed, the procedure was primarily applied to plants (Klein et al. 1988a, b, c; Wang et al. 1988) and thereafter transferred to other cellular systems including fungi. In several applications, an increase in transformation frequency and transformant stability was reported. In addition to these advantages, the simplicity of the method allows for a high number of transforming experiments to be undertaken in a short time, as both steps (plating of conidia and coating of microprojectiles with DNA) are simple and easy to standardize. It should also be noted, that this technique is the only one described so far for transformation of mitochondria and chloroplasts (Fox et al. 1988; Johnston et al. 1988; Ye et al. 1990).

2.7 *Agrobacterium Tumefaciens*-Mediated Transformation

Recently, a fundamentally different transformation technique has been reported. This was originally developed for plants, and involves the use of *A. tumefaciens* to deliver plasmid DNA into fungal cells (Bundock and Hooykaas 1996; Bundock et al. 1995; de Groot et al. 1998). *Agrobacterium* possess large plasmids (Ti-plasmids) and causes gall tumours at wound sites in infected dicotyledonous and monocotyledonous plants (Frommer and Ninnemann 1995; Hooykaas and Beijersbergen 1994; Linnemannstons et al. 1999; Van Veen et al. 1988; Zambryski 1992). This gall formation is due to the transfer of the T-DNA (part of the Ti-plasmid flanked by 24 bp imperfect direct repeats) into the plant nuclear genome. This transfer process strictly depends on the induction of virulence (*vir*) genes also located on the Ti-plasmid. Activation of the process is induced by products secreted from the wounded plant cells, such as acetosyringone (AS), (Winans 1992). An efficient plant transformation based on a Ti-plasmid system involves two plasmids working in a concerted action to transfer DNA to the respective host. One plasmid (Bin19) contains the T-DNA, the left and the right border regions of the Ti-plasmid, and a kanamycin resistance gene. The other plasmid bears all of the *vir* genes needed for T-DNA transfer. After transforming both vectors into *A. tumefaciens*, T-DNA is subsequently transferred to the plant by the aid of the *vir* gene products from the Ti-plasmid (Bevan 1984).

A similar system using a hygromycin B-resistance expression cassette as T-DNA and a selectable marker to detect transformation events has also been applied to filamentous fungi. Induction of the *vir* genes needed to induce the concerted transfer action of the binary vector system was achieved by supplementing the media with AS (de Groot et al. 1998). This method has been reported to give successful transformation of not only fungal protoplasts but also of conidia and hyphal tissue of *A. niger*, *A. awamori*, *Colletotrichum gloeosporioides*, *Fusarium venenatum*, *T. reesei*, *N. crassa*, and the mushroom *Agaricus bisporus* at varying transformation frequencies. In addition to ease of handling, this system may offer the potential to transfer high molecular weight DNA into fungal chromosomes as *A. tumefaciens* can insert at least 150 kb of foreign DNA to plant cells.

3 TRANSFORMING DNA

3.1 Transformation Vectors

In general, transformation vectors/plasmids are constructed for replication and selection in both *E. coli* and the respective filamentous fungus. Propagation in *E. coli* allows all standard DNA manipulations, and therefore the construction of suitable investigation systems. Several different types of vectors, such as basic plasmids, plasmids bearing *cos* sequences, yeast artificial chromosome (YAC) vectors and bacterial artificial chromosome (BAC) vectors have been used to transfer upto several 100 kb of foreign DNA into a selected fungus (Burke et al. 1987; Diaz-Perez et al. 1996; Gold et al. 2001).

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3.1.1 Autonomously Replicating Vectors

Fungal transformation vectors are basically divided into two different types: (a) autonomously replicating vectors and (b) integration vectors replicating within the fungal genome. In the case of autonomously replicating vectors, introduced sequences are recognized by the fungus and are thereafter replicated without integration into the genome. In addition to the autonomously replicating sequence (ARS) from *S. cerevisiae*, a number of such elements have been reported that confer autonomous replication to transformation vectors used for filamentous fungi, e.g., ANS1 and AMA1 of *Aspergillus* (Aleksenko and Clutterbuck 1995; Aleksenko et al. 1996; Gems et al. 1991), and telomere sequences of *Fusarium*, *Colletotrichum*, and *Cryptococcus*; (Edman 1992; Garcia-Pedrajas and Roncero 1996; Kistler and Benny 1992; Redman and Rodriguez 1994).

3.1.2 Vector Composition

Both functional and nonfunctional sequences are included into a vector system in order to mediate higher integration frequencies. Apart from the frequently reported use of homologous genomic DNA coding for selectable markers or regulatory sequences (e.g., promoters and terminators), the inclusion of nonselectable homologous sequences has been shown to increase transformation efficiency in some filamentous fungi. For example transformation and integration frequency in *T. harzianum* was notably improved by inserting a 2.4 kb fragment of the *T. harzianum* α-amylase gene into vector pAN7 bearing the *hph* marker gene conferring acetamide utilization (Herrera-Estrella et al. 1990).

3.1.3 Vector Propagation and Transformation Efficiency

A recent report has demonstrated on the influence of the vector amplification process. Transformation of *Colletotrichum* using either different *E. coli* strains or different single colony isolates of the same strain for vector propagation led to a high variability in transformation efficiency. The authors reported that 30 single colony derived cultures from *E. coli* strain DH5α produced equivalent quantities of vector varying in transformation frequency between 0 and 20,000 transformants/μg DNA (Redman and Rodriguez 1994).

3.2 Selectable Markers

The gain of dominant selectable phenotypes allows the selection of transformants within a background of non-transformed cells (Timberlake and Marshall 1989). Three different types of selectable markers are used for this purpose: (a) genes coding for suppressor tRNA, (b) auxotrophic markers, and (c) dominant selection markers.

A special type of selectable marker is the suppression of an auxotrophic mutation by tRNA suppressor genes. This was demonstrated in a *Podospora anserina* strain carrying the nonsense mutation *leu*1^{-}, and that was suppressed to leucine prototrophy by the tRNA suppressor genes *su*4-1 and *su*8-1 (Brygoo and Debuchy 1985).

The first transformations to be reported were carried out by converting auxotrophic mutants to prototrophy (Fincham 1989). Obviously, an indispensable prerequisite for a successful application of this selection method is the availability of appropriate mutants. Common mutants are those deficient in the metabolism of amino acids (leucine, arginine, and tryptophan), uracil, nitrogen, or in the catabolism of quinic acid. Vectors used for complementation contain the respective genes *leu*, *arg*, and *trp*, or *ura* and *pyr*, or *niaD*, or *qa2* [e.g., Alani et al. (1987); Baek and Kenerley (1998); Ballance et al. (1983); Banks and Taylor (1988); Berges and Barreau (1991); Case et al. (1979); Gruber et al. (1990); Penttila et al. (1987); Smith et al. (1991); Unkles et al. (1989); Van Hartingsveldt et al. (1987); Wada et al. (1996); Weidner et al. (1998); Yelton et al. (1984)].

Genes conferring resistance or a new metabolic activity have been widely used as dominant selection markers with several different species of filamentous fungi. This strategy, often followed as the isolation of auxotrophic mutants for many fungal organisms is difficult and changes to the genetic background may not be desirable, in many cases, e.g., industrially applied or plant pathogenic fungi.

The *hph* (hygromycin B phosphotransferase encoding) gene from *E. coli* is used as a marker in transformation by detoxifying hygromycin B, an amino glycoside antibiotic from *Streptomyces hygrosporicus* [e.g., Mach et al. (1994); Punt et al. (1987)]. Resistance to phleomycin, a metalloglykopeptide antibiotic causing DNA damage is conferred by the *ble* gene isolated from *E. coli* and *Streptoalloteichus hindustanus* by coding for proteins that bind to, and thereby inactivate phleomycin (Kolar et al. 1988; Mattern et al. 1988). Benlate (benomyl) resistance was gained by transforming both *N. crassa* (Orbach et al. 1986) and *A. nidulans* (May et al. 1985) with mutated genes encoding a benomyl resistant β-tubulin. The use of an oligomycin resistance gene (encoding ATP synthase) from an oligomycin resistant *A. nidulans* mutant has also been reported (Ward et al. 1986). G 418 resistance using a bacterial G 418 ^{R} can also be conferred to several fungi [e.g., Bull and Wootton (1984); Jimenez and Davies (1980); Penalva et al. (1985); Revuelta and Jayaram (1986)]. It shall be noted that in most cases these resistance genes have been fused to a variety of fungal promoter and terminator sequences to ensure expression.

A dominant heterologous expression marker useful with several filamentous fungi is the *A. nidulans amdS* gene. This codes for an acetamidase that allows growth on acetamide or acrylamide as sole carbon and nitrogen source. Such an activity is not observed in many wild-type fungal strains; thus, they are potential targets for transformation with this gene. Reports of successfully transforming various fungal species have been published, e.g., *A. niger* (Kelly and Hynes 1985), *Penicillium chrysogenum* (Beri and Turner 1987), *T. reesei* (Penttila et al. 1987), *Cochliobolus heterostrophus* (Turgeon

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et al. 1987), *Glomerella cingulata* (Rodriguez and Yoder 1987), and *T. harzianum* (Pe'er et al. 1991).

3.3 Markers for Two-Way Selection

Two-way selection systems are not only a useful tool to gain appropriate mutants, but also allow the reuse of the same selection system in several subsequent steps of genetic manipulation. Typical examples are uridine-negative mutants selected via loss of orotidine-5′-monophosphate carboxylase activity (encoded by *ura3* or *pyr4* and required for uridine biosynthesis) which confers resistance to the inhibitory analogon 5-fluoro-orotic-acid (Alani et al. 1987; Berges and Barreau 1991; Gruber et al. 1990; Smith et al. 1991). The nitrate reductase gene (e.g., *niaD* of *A. nidulans*) is also an attractive selection marker for developing a gene transfer system in genetically poorly characterized species. In this case, loss of *niaD* function can be selected for by the gain of resistance to chlorate (Unkles et al. 1989). Furthermore, the *amdS* marker gene described above can be used in such a two-way selection by screening for fluoro-acetamide resistance versus acetamide utilization (Debets et al. 1990).

Recently, such a system has successfully been used to clone a transcriptional regulator of *A. niger* which will be discussed in more detail later.

3.4 Cotransformation

Cotransformation, where recipient cells are treated with two different kinds of DNA, is one way of overcoming the problem that in most cases transforming genes cannot be directly selected for. To accomplish this, transformation is undertaken together with a more readily selectable marker, as it is known that the probability of taking up both transforming DNAs is quite high (Fincham 1989). Although cotransformation frequencies for different fungi have been reported to vary from <10 to 100%, the majority of fungi show rates higher than 50% [e.g., Cooley and Caten (1989); Harkki et al. (1991); Kubicek-Pranz et al. (1991); Nicolaisen and Geisen (1996); Penttila et al. (1987); Punt et al. (1987)].

Cotransformation was first investigated by Wernars et al. (1987), who reported that the uptake of both transforming DNAs can be increased if the ratio of cotransforming to transforming DNA is kept high. Reported cotransformation frequencies vary widely and seem to be dependent on both the organism and the cotransformation conditions.

For some species, the frequency of cotransformation has been demonstrated to be dependent on the conformation of both transforming DNAs. For example cotransformation in *Phytophtora infestans* occurred in only 10% of transformants when circular DNA was used but reached nearly 100% when linearized DNA was used (Judelson 1993).

It should be noted that nonselectable DNA often recombines with selectable vector DNA prior to either integration into the fungal genome or autonomous replication of the vector during cotransformation (Gems and Clutterbuck 1993; Judelson 1993).

4 PURIFICATION AND CHARACTERIZATION OF TRANSFORMANTS

4.1 Integration of DNA into the Genome

Except of the few examples of autonomously replicating systems, all commonly used vectors integrate into the genome of the respective filamentous fungus being transformed. Analyzing the chromosomal DNA of such transformants shows that, in general, three types of integration events occur: (a) vector integration by homologous recombination, (b) ectopic integration by nonhomologous recombination, and (c) gene replacement. Detailed descriptions of these integration types have been described elsewhere [e.g., Fincham (1989); Mach and Zeilinger (1998)].

The occurrence of any particular integration type is highly variable and seems to be dependent on the species to be transformed, the transformation technique, and probably mainly on the conformation of the transforming DNA (circular or linear).

Frequencies of homologous integration (I) events are highly variable for different species, e.g., *A. nidulans* about 80% (Yelton et al. 1984), *N. crassa* 1–5% (Case 1986), or *Hypocrea jecorina* about 2% (Mach et al. 1995; Seiboth et al. 1992).

For ectopic integration (II) it is believed that small stretches of target-vector DNA identity may provide nucleation sites for the initiation of a recombination event, ectopic integration (II). The occurrence of such sites in the genome would then strictly depend on their sizes and will lead to random integration (Timberlake 1991). In contrast to this, Dialinas and Scazzocchio (1989) demonstrated that ectopic plasmid integration into the genome of *A. nidulans* has clear site preferences. Mechanisms involved in the phenomenon of ectopic integration are still unclear and warrant further investigation.

Gene replacement (III) takes place when the target gene is apparently replaced by the introduced copy but none of the vector sequence is additionally integrated into the genome. Such a gene conversion has been shown to be favored by the use of linear molecules on a number of occasions [e.g., Aramayo et al. (1989); Boylan et al. (1987); Fowler and Brown (1992); Mach et al. (1995); Seiboth et al. (1992)].

Multicopy integration of vectors by tandemly repeated copies is widely observed in filamentous fungi. Several explanations for this observation can be provided: (a) a first step of integration of a single vector copy into the homologous locus may be followed by successive rounds of further homologous integrations, (b) an ectopically integrated plasmid may function as a target for further tandem integrations, and (c) plasmids extrachromosomally concatemerize via homologous recombination followed by

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integration into the genome of these circular oligomers (Fincham 1989).

It should be noted that in *A. nidulans*, for example, tandem repeat sequences are sometimes lost after cell fertilization or outcrossing (Tilburne et al. 1983). Elimination of duplicate sequences is reported at very high frequencies for *N. crassa* by the so-called RIP process (repeated induced point mutation) (Selker and Garrett 1988; Selker et al. 1987).

When studying genes, ectopic integration may also lead to problems as both positioning effects and disruption or modification of resident genes may cause misinterpretations. This problem may be overcome by using either targeted integration systems (integration of vectors by incorporating homologous selectable markers or non selectable DNA sequences, see above) or by studying a statistically relevant number of transformants.

4.2 Purification of Mitotically Stable Transformants

Since cells from some filamentous fungi are multinucleate, most derived protoplasts also bear more than one nucleus. Furthermore, several fungal species have multinucleate conidia. Subjecting such material to transformation will consequently lead to heterokaryons, as commonly not all nuclei of one cell compartment will take up the DNA. Indispensable prerequisites, for obtaining homokaryotic recombinants are therefore subsequent steps of genetic purification following transformation. If fungal species form uninucleate conidia, (e.g. *Aspergillus* spp.), purification can be simply achieved by plating conidia and isolating single colonies. For polynucleate systems, purification can be undertaken by a similar procedure, but at least three plating rounds will be needed to give a high probability of a stochastic loss of one or other nucleus (Fincham 1989).

If selective conidiation steps are interchanged with non selective ones, non stable transformants, e.g., those bearing non integrated vectors, will be lost, leading finally to homokaryotic mitotically stable strains.

5 APPLICATION IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY

Transformation systems are basic tools for molecular biology based research and for strain improvement for biotechnology. The following section will outline a few examples of the application of transformation systems with special emphasis on recent novel developments. Additional information on further applications and uses such as, overexpression of homologous and heterologous genes and improvement of industrial strains has been described elsewhere (e.g., Gold et al. (2001); Goosen et al. (1992); Hynes (1996); Mach and Zeilinger (1998); and citations therein).

5.1 Cloning by Complementation

Cloning by complementation requires respective mutants and the construction of genomic or cDNA libraries. Furthermore, the success of such strategies is strictly dependent on the transformation frequency and ease of selection of the complemented phenotype. This strategy has therefore often been used to isolate genes involved in cell metabolism, such as those involved in the biosynthesis of amino acids and pyrimidines, in nitrogen assimilation, and fungicide resistance. For many genes (e.g., nonnutritional) no direct selection is possible or it is too time consuming and labour intensive, and so complementation techniques have been developed for such genes. Cloning of regulatory genes involved in gene expression is often hampered by cross feeding in traditional selection methods. Recently, van Peij et al. (1998) developed a cloning system in *A. niger* based on the bi-directional selection marker *pyrA,* that codes for orotidine-5′-phosphate decarboxylase which catalyses a step in pyrimidine nucleotide synthesis. In the selection method used, *pyrA* is under the control of an *in cis* acting element of a regulated xylanolytic gene. Introducing this selection cassette into *A. niger* allowed a pathway-specific *pyrA* expression and bi-directional selection, i.e., uridine prototrophy for a PYR^+ phenotype or 5-fluoro-orotic acid resistance for a PYR^- phenotype. Mutants for positively and negatively pathway-specific *in trans* acting factors could be selected for in this way.

5.2 Restriction Enzyme Mediated Integration (REMI)

Gene disruption is the most frequently used alternative to complementation cloning. The earliest attempts were undertaken with occasional ectopic integrations of homologous vector systems, resulting in gene disruptions in the mutants generated (Diallinas and Scazzocchio 1989). Transposons have also been exploited to create insertion mutations with great success in many bacterial systems but these have rarely been used in eukaryotic systems. Transposons are not found in all fungi and particular laboratory and industrial strains often lack such elements. The so-called REMI mutagenesis was first developed in *S. cerevisiae*, an organism in which homologous integration is extremely frequent (Schiestl and Petes 1991). Transforming *S. cerevisiae* with nonhomologous DNA linearized by digestion with a restriction enzyme and then including this enzyme into the transformation mixture prompted the integration of the transforming DNA into the respective restriction sites in the genome. The mechanism of REMI is currently not fully understood and possible explanations are discussed elsewhere (Manivasakam and Schiestl 1998; Riggle and Kumamoto 1998; Thode et al. 1990). It should be mentioned that the main mechanism occurring during REMI

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is thought to be a form of nonhomologous end-joining (Thode et al. 1990).

Since the first use of REMI in *Dictyostelium discoideum* (Kuspa and Loomis 1992), this method has also been applied to a variety of Asco- and Basidiomycetes, including *C. heterostrophus* (Lu et al. 1994); *Ustilago maydis* (Bolker et al. 1995); *Magnaporthe grisea* (Liu et al. 1998; Sweigard et al. 1998); *Coprinus cinereus* (Granado et al. 1997); *Candida albicans* (Brown et al. 1996; Riggle et al. 1997); *A. fumigatus* (Brown et al. 1998); *A. nidulans* (Sanchez et al. 1998); *Lentinus edodes* (Sato et al. 1998); *A. niger* (Shuster and Bindel Connelley 1999); *Alternaria alternata* (Tanaka et al. 1999); *Giberella fujikuroi* (Linnemannstons et al. 1999); *A. oryzae* (Yaver et al. 2000); *C. graminicola* (Thon et al. 2000); *Hansenula polymorpha* (van Dijk et al. 2001) and *F. oxysporum* (Namiki et al. 2001).

REMI has several advantages as a method for genetic analysis: (a) physically tagged random insertion mutations, (b) stimulation of transformation frequency of several folds, and (c) creation of single, stable, and un-rearranged genomic insertions. In addition to insertion mutagenesis, REMI has also been used in new applications such as RFLP mapping (Kuspa and Loomis 1994; van Dijk et al. 2001), promoter trapping (Chang et al. 1995), promoter tagged REMI (Shuster and Bindel Connelley 1999), and dominant genetics (Brown et al. 1996). It should be noted that REMI transformation protocols are developed empirically and that the form of transforming DNA (linear or circular) and the restriction enzyme used varies from species to species. Stimulation of transformation (from non to 20-fold) as well as type of integration also varies widely between different organisms.

5.3 Use of Reporter Genes for Promoter Analysis

Reporter genes have extensively been used to characterize regulatory DNA sequences from bacteria to higher eukaryotes. The main advantage of such systems is the easy investigation of recombinant strains bearing such reporter systems by detection of the activity gained from the reporter gene insertion. The most prominent reporter genes, the β-galactosidase (*lacZ*), the β-glucuronidase (GUS), the β-lactamase (*bla*), the glucose oxidase (*goxA*) and the green fluorescent protein (*gfp*) can be used in systems where the respective transformants produce pigments that allow the detection and quantification of the inserted genes. In contrast to other systems that require cultivation media containing substrates for the detection of the expressed enzymes, no such substrates are required for the autofluorescing protein *gfp* and transformants may be visualized by epifluorescence (Prasher et al. 1992). *Gfp* has been used extensively in a number of studies including the investigation of nuclear traffic in fungal hyphae in *A. nidulans* (Suelmann et al. 1997), identification of a motor protein in *U. maydis* (Lehmler et al. 1997), regulation of biocontrol specific genes in *T. atroviride* during host interaction (Zeilinger et al. 1999), compartmentation of the glucose repressor Cre1 in *Sclerotinia sclerotiorum* (Vautard et al. 1999), protein secretion in *A. niger* (Gordon et al. 2000), subcellular localization of the purine transporter in *A. nidulans* (Valdez-Taubas et al. 2000), identification of components involved in polarized growth in *Ashbya gossypii* (Alberti-Segui et al. 2001), and subcellular localization of homocitrate synthase in *P. chrysogenum* (Banuelos et al. 2002). The broad range of applications of GFP is due mainly to the fact that this system is non-toxic and non-invasive to the cell and can be studied *in vivo*. In the future, the use of fluorescent proteins is likely to increase, particularly as various recombinant versions that emit light at other wavelengths (e.g., blue, red, yellow) are now commercially available.

6 CONCLUSIONS

Significant progress has been made in transforming a great variety of different filamentous fungi during the last decade. This success has largely been due to the development of novel techniques such as the particle bombardment and *Agrobacterium*-mediated transformation, which either strongly increased low transformation frequencies or made former "nontransformable" fungi accessible to gene transfer. On the other hand, the fate of the transforming DNA in the respective organisms is still not fully understood and recombination and re-arrangements are often observed. These facts still limit the applicability of such transformation systems and often make interpretations of the results observed difficult. Finally it should be noted that a reasonable number of different markers for transformation are already in use, but, with a few exceptions, no tools such as targeted integration systems, artificial chromosomes, or centromere vectors are yet available.

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10

Genetic Manipulation Systems for Nonconventional Fungi

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1 INTRODUCTION

The term fungi is used for describing a life style rather than as a systematic category. In this phenomenological, ecological sense, fungi are nonphotosynthetic organisms that propagate via spores. Most of them are chemo-osmotrophs with the exception of slime moulds that are phagotrophs. Fungi are primary heterotrophs with the possible exception of oomycetes, which at the sequence level form a common clade with algae. In this classical broader sense fungi are certainly polyphyletic and range somewhere between algal heritage (Oomycota), protozoa (Myxomycota, Acrasiomycota), and the Mycota *sensu strictu* (Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota). Only this latter group, Mycota or Eumycota is monophyletic and has been recognized in recent years as the natural sister group of Metazoa. In this sense the terms fungi and (Eu)Mycota are not synonymous. Due to their polyphyletic origin, fungal biological diversity is necessarily very high; consequently their genome organization and the concepts and procedures for genetic handling and manipulation are not easily comparable. The wealth of physiological diversity in fungal organisms becomes evident from their basal position in the tree of life.

Even within the monophyletic Mycota with the phyla Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota, and maybe, although not strictly supported by unequivocal data, a fifth one, Glomeromycota (formerly: Glomales, Zygomycota; Schüssler et al. 2001), the genetic heterogeneity is very high. A closer look at basic features of genome organization makes this clear. Whereas asco- and basidiomycetes have essentially streamlined genomes with small genome sizes and little repetitive DNA, zygomycetes and *Glomus*-like fungi have a strong tendency towards larger genomes with typically 35% repetitive DNA (Dusenbery 1975; Wöstemeyer and Burmester 1986; Wöstemeyer and Kreibich 2002). Unfortunately very little is known about the basic genomic parameters of chytridiomycetes (Ojha et al. 1977) that are believed to range phylogenetically at the basis of Eumycota.

Merely descriptive terms, like filamentous fungi or yeasts, do not help very much in predicting genetic or metabolic properties of biotechnically interesting fungi. The ability of growing as a mycelium has been realized in all phyla. In addition, yeast/mycelium dimorphism within a single species is common in many organismic groups. There is no way to predict genetic or biochemical properties over the border of phyla based on simple phenomenological criteria. Indeed, phenomenological categories do not help at all. Despite modern molecular fingerprinting techniques, there is still no logical rationale for a reasonable forecast of metabolic properties. However, in the long run, there are realistic chances for developing reliable probes for defined biosynthetic pathways. Also in this sense, a concept of looking at the fungal world that is strictly based on phylogenetic rationale would be helpful and necessary.

Apart from providing basic information on strain manipulation and improvement by DNA-mediated transformation, protoplast fusion and, where applicable, by natural horizontal gene transfer, this report intends to focus the attention of the scientific community on unconventional fungi with novel properties. A wealth of phylogenetic novelties and, consequently, new realms of biotechnical applications will be found, be it with respect to novel metabolites or with respect to novel properties in heterologous gene expression, protein modification, and secretion.

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2 MYXOMYCOTA

2.1 General and Applicative Aspects

Myxomycetes are diploid plasmodial slime molds. In contrast to true fungi (Eumycota) they are phagotrophs feeding on bacteria and small eukaryotes. Phylogenetically their position in the tree of life is a matter of debate. They probably range near the basis of eukaryotic life and may well be interpreted as an independent kingdom. They have highly differentiated developmental pathways towards the formation of fruiting bodies in which spores for the propagation of these organisms are formed. The accepted model system for studying morphogenesis, cytoplasmic movement, and the mechanics of the cytoskeleton is *Physarum polycephalum*. One of the most attractive features of this organism is the highly synchronized mitosis in plasmodia. Millions of nuclei enter division at the same time, rendering *P. polycephalum* as the best model system for studying DNA replication in eukaryotes. The type of signal that coordinates the nuclei is totally unclear and it will be necessary to consider physical parameters more than chemical ones.

The morphological diversity of fruiting bodies and the wealth of pigments that these organisms synthesize have attracted chemists to look into the biotechnical potential of myxomycetes. A carbohydrate binding protein, physarumin, was isolated from *P. polycephalum*, and was shown to inhibit the growth of several tumor cells and to induce apoptosis in mouse leukemia P388 cells (Numata et al. 1998). In addition the alkaloid arcyriacyanin A, an indole pigment from the slime mould *Arcyria obvelata*, affects tumor growth, probably by inhibiting protein kinase C and protein tyrosine kinase (Murase et al. 2000). *Fuligo septica* produces bioactive substances that in addition to inhibiting cellular growth without being cytotoxic, exert antibiotic activity towards gram-positive bacteria and the human-pathogenic yeast *Candida albicans* (Chiappeta et al. 1999). There are also considerations to exploit myxomycetes for producing the neurotransmitter precursor DOPA (3,4-dihydroxyphenyl-alanine). It is possible to extract DL-DOPA from differentiating plasmodia of *Stemonitis herbatica* (Loganathan 1998; Loganathan and Kalyanasundaram 1999); the slime mould uses DOPA as precursor for melanin, which forms the major pigment of its sporangia. Many myxomycetes produce a highly anionic polyester of malate, poly-β-L-malate that is considered as a raw material for the chemical industry (Rathberger et al. 1999).

2.2 Tools for Genetic Analysis

The main targets of genetic analysis are the transition from uninucleate amoebae to multinucleate plasmodia and the development of fruiting bodies. Amoebae can be transformed stably with plasmids by electroporation (Burland and Bailey 1995). Appropriate vectors rely on the bacterial *hph* gene for hygromycin phosphotransferase under the control of the homologous actin promoter (Burland et al. 1993). Promoter regions and elements functioning as origins of replication seem to map to identical genomic regions as has been shown for a 1 kb region upstream of the *ardC* actin gene of *P. polycephalum* (Pierron et al. 1999). Typically, transformants carry a single copy of the introduced DNA at an ectopic integration site. Transformation followed by homologous gene replacement can also be achieved with reasonable frequencies in 5% of all transformants if linear plasmid DNA is used (Burland and Pallotta 1995). Today, genetic analysis has reached a stage, where developmental genes are identified, cloned, and analyzed at the molecular level (Bailey et al. 1999).

3 ACRASIOMYCOTA

3.1 General and Applicative Aspects

This group of slime moulds differs from Myxomycota or true slime moulds by the cellular, uninucleate organization, and by the haploid character of the trophic form. The life style, however, is similar. Amoebae aggregate to pseudoplasmodia and live as phagotrophs. The phylogenetic relationship with plasmodial slime moulds is not clear, but the Acrasiomycota too range somewhere around basic protist-like eukaryotic organisms. The model organism is clearly *Dictyostelium discoideum* that has been studied for a long time with respect to surface interaction during amoebal aggregation and subsequent fruiting body formation, and with respect to the action of various small signal molecules that trigger this process and cAMP is only one of them. The genomic sequence of *D. discoideum* is near to completion; major parts are already published (Glöckner et al. 2002).

Whereas *D. discoideum* is a widely accepted model organism for basic biological phenomena, cellular slime moulds have found comparably marginal biotechnical interest. They may play some role in obtaining access to unsaturated fatty acids; a δ-5-fatty acid desaturase was identified in *D. discoideum* (Saito and Ochiai 1999). *Dictyostelium* has been shown to represent a promising expression system, especially for heterologous synthesis of glycoproteins (Heikoop et al. 1998; Jung and Williams 1997; Williams et al. 1995). Apart from the glycosylating properties of the organism, the wealth of tools for genetic manipulation renders *Dictyostelium* highly suitable for molecular biotechnology.

3.2 Tools for Genetic Analysis

The literature on molecular techniques for analysis and manipulation cannot be covered in depth. *D. discoideum* was shown to be transformable very early by vectors conferring aminoglycoside antibiotic resistance, either under the control of the homologous actin promoter (Hirth et al. 1982) or driven by the SV40 promoter (Barclay and Meller 1983). Since then,

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a wealth of different vectors, including those based on naturally occurring plasmids from *D. discoideum* (Chang et al. 1990; Leiting and Noegel 1988), various selectable markers (Chang et al. 1991; Egelhoff et al. 1989; Sutoh 1993) and methods for introducing the DNA (Knecht and Pang 1995; Wetterauer et al. 2000) have been developed and exploited for studying the differentiation program from unicellular amoebae to sporangia, which are irreversibly differentiated into stalk and spore cells.

A very attractive feature of the *Dictyostelium* system is the ease by which antisense RNA approaches can be used to study gene expression. The antisense concept has been recognized as a naturally occurring regulatory principle (Nellen et al. 1992) and was immediately exploited for studying cellular development (Fang et al. 1991) and identification of genes (Spann et al. 1996) by artificial antisense constructs. Additional valuable tools for genetic analysis are restriction enzyme mediated mutagenesis (Kuspa and Loomis 1994; Riggle and Kumamoto 1998) and PCR-mediated gene disruption (Kuwayama et al. 2002). These and related approaches are highly advanced and contribute to rendering *Dictyostelium* as one of the best understood eukaryotic systems.

4 OOMYCOTA

4.1 General and Applicative Aspects

In contrast to true fungi (Eumycota) oomycetes are diploid organisms with a special type of sexual development, oogametangiogamy. Phylogenetically they are related more to algae than to fungi, a kinship that is now generally accepted, and which is supported by several molecular markers (Baldauf et al. 2000; Voigt and Wöstemeyer 2001). There are many plant pathogens in this organismic group, including the highly important *Phytophthora* and *Pythium* species, causative agents of potato late blight (*Phytophthora infestans*), bent grass red blight (*P. aphanidermatum*), and many other crop diseases. Especially the communication between host and oomycetous parasites via elicitor substances is an established field of research in phytopathology (Boissy et al. 1996; Nespoulos et al. 1999). Elicitins, small proteins secreted by the pathogens, and other substances induce hypersensitive reactions on the host plants, and thus are important components of the signal pathway leading to pathogen recognition by the plant and finally to resistance. The general relevance of proteinaceous elicitins that seem to act via the formation of sterol conjugates for plant-oomycete interaction has recently been reviewed (Ponchet et al. 1999).

Biotechnically, oomycetes have attracted some attention with respect to the synthesis of polyunsaturated fatty acids, especially eicosapentaenoic acid (20:5n-3) and arachidonic acid (20:4n-6) which in the case of the marine oomycete *Haliphthoros philippinensis* may constitute up to 19 and 21% of total fatty acids, respectively. Under optimal fermentation conditions, the amount of eicosapentaenoic acid can be increased to 27% of total fatty acids, corresponding to more than 300 mg/l culture volume (Kim et al. 1998). Similarly encouraging results were obtained with other oomycetes, *Pythium debaryanum* (Galanina et al. 1999) or *P. irregulare* (Walker et al. 1999).

Another field of interest is the ability of several oomycetes to infect and destroy insects. The most intensively studied oomycetes are *Achlya ambisexualis* and *Saprolegnia* sp. both are watermolds and occur naturally on flies and freshwater fish. It has been proposed to use such organisms as control agents for insects that constitute vectors of infectious diseases. Promising candidates are *Leptolegnia caudata* or *Aphanomyces laevis*, both parasitising larvae of the malaria vector *Anopheles culicifacies* (Bisht et al. 1996), or the mosquito pathogen *Lagenidium giganteum* (Bell et al. 1989).

Many oomycetes live preferentially in highly polluted water and are, therefore, supposed to harbor a variety of degradative capabilities. Novel species from such locations are currently being described (Steciow and Eliades 2002), the biotechnical potential of which is open for discovery.

Introducing natural isolates into biotechnical application requires tools and methods for strain improvement. Due to the phytopathological relevance of oomycetes, a variety of *in vivo* and *in vitro* methods are available for pathogens from the genera *Phytophthora* and, to a limited extent, *Bremia*. A variety of genes from oomycetes and some basic genetic tools including regulatory sequences for expressing genes in oomycetes (Judelson et al. 1992) are available. The *NiaA* gene from *P. infestans* encoding nitrate reductase has been cloned and functionally identified by heterologous expression in a defective mutant of *Aspergillus nidulans* (Pieterse et al. 1995). Another characterized gene codes for the heat shock protein Hsp70 and stems from *Bremia lactucae* (Judelson and Michelmore 1989). A recent major achievement that will be of help for functional analysis of the *Phytophthora* genome is the construction of a genomic library in bacterial artificial chromosomes (BAC) with insert lengths above 100 kb. The authors have shown that these constructs could be used to retransform *P. infestans* (Randall and Judelson 1999). The bacterial *npt* gene conferring aminoglycoside (G418) resistance was employed as a selectable marker. Transformation efficiency seems to be higher with BACs than with smaller plasmids. This should open the field for detailed functional expression studies and recombination analysis in an important pathogen.

4.2 *In Vivo* Tools

Applicable techniques for protoplast formation were developed very early (Bartnicki-Garcia and Lippman 1966). Consequently, heterokaryons and recombinant genotypes were constructed by protoplast fusion and a speciality in oomycetes research by direct transfer of nuclei. Especially direct nuclear transfer has proven to be a powerful tool of genetic analysis. Artificial heterokaryons were shown to exhibit increased vigor (Gu and Ko 2001). The same

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technique may be applied for constructing interspecific hybrids between the species *Phytophthora capsici* and *Parasitella parasitica* (Gu and Ko 2000). The system is valuable for studying communication in trans between genetically different nuclei within a single mycelium (Van West et al. 1999a,b). Apart from establishing defined heterokaryons, nuclear grafting allows the construction of genetic recombinants via a parasexual cycle that occurs in reasonable frequencies (Gu and Ko 1998).

4.3 Transformation Systems

Transformation of oomycetes can be achieved by similar approaches as shown for ascomycetous fungi, as long as homologous regulatory sequences are used to drive the genes used as selective markers. The aquatic oomycete *A. ambisexualis* was transformed as early as 1988 by Manavathu et al. (1988). The system used a G418 resistance gene under control of the SV40 promoter. By this simple approach transformation efficiency was low, and the vector molecules were heavily rearranged. In addition, the major important plant-pathogenic *Phytophthora* species turned out to be transformable fairly easily (Bailey et al. 1991; Judelson et al. 1991; 1993). Normally protoplasts are used as cellular targets that take up DNA deliberately in the presence of polyethylene glycol and Ca^{2+} ions, sometimes aided by lipofectin (Bottin et al. 1999). *Saprolegnia monoica* was transformed to hygromycin resistance (Mort-Bontemps and Fèvre 1996) by vectors that contained the bacterial phosphotransferase gene under control of promoter regions from the *Hsp70* gene or the *Ham34* gene of *Bremia lactucae* (Judelson and Michelmore 1991). Foreign DNA is normally established ectopically by single or multiple copy integration (Judelson et al. 1993; Mort-Bontemps and Fèvre 1997). Unfortunately, the fate of foreign DNA after integration into the chromosomal complement of the recipient cannot be predicted completely, at least not at the level of gene expression. Transgenes may become silent (Judelson and Whittaker 1995; Mort-Bontemps and Fèvre 1997). The reasons for this behavior are not understood; deletions, point mutations, and methylation could be excluded (Judelson and Whittaker 1995).

Recently, the introduction of the bacterial *UidA* gene encoding β-glucuronidase (GUS), and, more conveniently, the *GFP* gene from the jellyfish *Aequoria victoria* into transformation vectors opened the possibility for studying temporal and local expression patterns *in vivo* and *in situ*. By a GUS approach it was elegantly shown that the *ipiO* gene from *P. infestans* was expressed predominantly during the infection process in subapical and vacuolated tip areas of invading hyphae, and not in zoospores, cysts, and young mycelia (Van West et al. 1998). Bottin et al. (1999) used the green fluorescent protein as reporter system for following spatial distribution of the pathogen *P. parasitica* in infected tobacco plants. A similar approach was followed for the wide host range pathogen *P. palmivora* (Van West et al. 1999a, b).

5 CHYTRIDIOMYCOTA

5.1 General and Applicative Aspects

Modern chytridiomycetes are believed to resemble most closely to the ancient forms of Eumycota by most of the authors. They are the only eumycotal division that has retained flagella. Therefore, this group marks the transition from water to land-inhabiting fungi. Ecologically they are highly important destruents of cellulolytic materials. Digestion of plant material in ruminants would not be possible without anaerobic chytridiomycetes. The cellulolytic enzymes especially of the genus *Neocallimastix* have been studied in detail, including heterologous expression studies (Ekinci et al. 2002).

Chytrids are studied predominantly with respect to basic physiological and cell-biological phenomena. Accepted model organisms are *Allomyces* spp. and *Blastocladiella emersonii*. The mechanisms underlying zoospore formation and germination, sexual differentiation and sexual hormone action (Pommerville 1977), cytoskeleton function (Lowry et al. 1998; McDaniel and Roberson 2000), and also the role of anaerobic chytridiomycetes for cellulose digestion in the rumen microflora (Leschine 1995) were studied in detail, but the transition to biotechnical application has not been tried. Exploiting chytrids for biotechnical performance is only in the very beginning and the potential is evident. Apart from cellulolytic enzymes some interesting proteases, alkaline serine proteases (Ojha 1996), and Ca^{2+} stabilized proteases were described in these fungi (Krarup et al. 1994; Ojha et al. 1999).

5.2 Tools for Genetic Analysis

The genetic level of chytrid analysis requires development of adequate molecular tools. Basic genomic characteristics are only marginally known (Ojha and Turian 1971; 1977; Ojha et al. 1977), and methods for *in vitro* genetic manipulation of chytrids have not yet been developed. There are, however, valuable tools for studying gene expression. The promoter or control region of an enolase encoding gene from *Neocallimastix frontalis* was cloned and sequenced. The difference to fungal promoters from ascomycetes is intriguing (Fischer et al. 1995). Until now, these fungi have been analyzed predominantly under phylogenetic aspects. In this field our knowledge is considerable, also at the DNA sequence level. Among other sequences that might serve as sources for promoters and other signal elements for gene expression, the complete mitochondrial DNA sequence of *Allomyces macrogynus* has been elucidated (Paquin and Lang 1996). The sequence analysis provided evidence for a recent parasexual transfer of functional mitochondrial genes, a subunit of the ATP synthetase complex and an endonuclease gene, to *A. macrogynus* that is not found in a close relative, *A. arbusculus* (Paquin et al. 1994). If chytridiomycetes have a general tendency towards lateral gene transfer, powerful

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strategies for genetic analysis and genetic manipulation could be developed on this basis. Double-hairpin structures in mitochondrial DNA led to the assumption of mobility of such elements (Paquin et al. 2000). The underlying enzymatic apparatus could form the basis of genetic manipulation systems, at least in mitochondria. Unfortunately, the analysis of the nuclear genome is lagging behind.

6 ZYGOMYCOTA

6.1 General and Applicative Aspects

Zygomycetes, especially from the order Mucorales, are valuable model organisms for studying various basic biological phenomena. Sexual recognition and mating type differentiation has been studied at the physiological and partially at the genetic level, especially in *Mucor mucedo*, *Phycomyces blakesleeanus*, and *Absidia glauca*. *Mucor racemosus* serves as a suitable model for elucidating the molecular and physiological basis of yeast/mycelium dimorphism. The best studied system for understanding the effect of light on growth regulation and on carotene formation is *P. blakesleeanus* (Arrach et al. 2001; Blasco et al. 2001; Sanz et al. 2002), similar investigations on the regulation of carotene biosynthesis have been made in *Mucor circinelloides* (Navarro et al. 2001), and *A. glauca* has the advantage of the best techniques for genetic manipulation (Schilde et al. 2001).

Biotechnical interest in zygomycetes is based on many applications. A huge market based on zygomycetes is the production of regio- and stereospecifically hydroxylated steroids for antiinflammatory drugs and contraceptives. Comparable with respect to market volume is the fermentation of aspartate proteases with rennin-like properties for cheese production. β-carotene is most efficiently produced by means of *Blakeslea trispora* (Jeong et al. 2001). With respect to requirements of the food industry this is a rapidly growing market. In addition with respect to their cell wall component, chitosan which is technically produced in large scale by deacetylation of crab shell chitin, zygomycetes are being considered as an alternative (Jaworska and Konieczna 2001).

The potential of zygomycetes is based less on secretion of bioactive metabolites than on their intrinsic enzymatic properties. Many zygomycetes are known to contain considerable amounts of polyunsaturated fatty acids, especially eicosapentaenoic acid (Bajpai et al. 1991) and arachidonic acid (Steekstra 1997) in *Mortierella alpina*. There are also observations that *M. verticillata* produces lactone compounds that induce human LDL receptors in tissue culture (Dekker et al. 1998). Most efforts for developing genetic transformation systems in mortierellalian fungi are motivated by directing the fungal metabolism towards synthesis of more or specially designed fatty acids for nutritional and technical purposes (Mackenzie et al. 2000). Zygomycetes were shown to degrade pentachlorphenol (Seigle-Murandi et al. 1991) and especially *Rhizopus oryzae* was found to dechlorinate and to detoxify bleach plants effluents more efficiently and at lower cosubstrate concentrations than previously described for basidiomycetes (Nagarathnamma and Bajpai 1999).

Zygoymcetes share a variety of positive features that make them attractive for biotechnical applications. Typical beneficial properties comprise vigorous growth, low nutrient requirements, no toxin secretion, few human, animal or plant pathogens, few natural auxotrophies in most taxa of mucoralean and mortierallalean fungi, the availability of biochemical abilities encountered exclusively in this fungal group, and essentially no difficulties with instability of strains due to sectoring of colonies.

In the future, there will be urgent needs for improvement of industrial strains by several complementary approaches, conventional mutagenesis, *in vitro* genetic manipulation, and improvement of fermentation and downstream processing.

6.2 *In Vivo* Tools

Strain improvement of all zygomycetous fungi is severely hampered by the inefficiency of the sexual cycle. Most technically relevant mucoralean zygomycetes form sexual zygospores deliberately, but germination rates range at best around 1%, and in some species the conditions for germination under laboratory conditions are not known at all. For improvement of technical strains conventional genetic crosses are essentially irrelevant. Apart from low germination rates the processes determining the fate of individual nuclei in the multinucleate zygosporangia, the frequency of nuclear fusions and meiotic recombination events are not really understood. It is even possible that some mucoralean species do not undergo ordered meiosis. In this respect an analysis by Mehta and Cerdá-Olmedo (2001) on the assumed formation of intersexual partial diploids in *Phycomyces* following crosses of compatible strains gives rise to doubts in conventional interpretations of the sexual cycle in zygomycetes. The clearest data for *P. blakesleeanus* date back to 1975 (Eslava et al. 1975). The authors found that approximately 80% of all progeny from sexual crosses can be traced back to a single nuclear fusion event in the multinucleate zygospore. The mechanistic basis for the residual 20% of recombined offspring could not be revealed. It is also not clear if the *Phycomyces* data have model character for the Mucorales.

Mucoralean zygomycetes can be protoplasted fairly easy if the peculiarities of cell wall composition are taken into account. The primary cell wall chitin is deacetylated to chitosan during hyphal growth. Consequently optimal protoplasting rates are achieved if the enzyme cocktail contains reasonable amounts of chitosanase (Wöstemeyer and Brockhausen-Rohdemann 1987). Regeneration rates are usually high; regenerates normally form hyphae directly from the protoplast or they may first form a syncytial irregular mass, as has been shown for *Benjaminiella poitrasii* (Chitnis and Deshpande 2002). Protoplast fusion between mating types has shown that sexually complementary nuclei are compatible within the same mycelium and give rise to

homothallic derivatives in *A. glauca*. Parasexual recombinants occur with reasonable frequency. Recombinants that combine with the complementary mating types in a single nucleus are also homothallic (Wöstemeyer and Brockhausen-Rohdemann 1987; Wöstemeyer et al. 1990). In heterokaryons, the system allows to study trans-acting regulatory phenomena (Wöstemeyer et al. 1990). A review on the potential of protoplast fusion approaches has been recently published (Wöstemeyer and Wöstemeyer 1998).

Mucoralean species belonging to the broad host range of the facultative fusion parasite *P. parasitica* offer the possibility for a different parasexual manipulation pathway. The infection cycle includes a stage where a small compartment containing approximately 20 nuclei of the parasite fuses with the host mycelium. The parasite's nuclei enter the host and undergo recombination events. By this unique parasexual system the complementation of auxotrophies of the host by genetic information from the parasite has been shown (Kellner et al. 1993; Wöstemeyer et al. 1995; 2002). If *P. parasitica* is transformed with autonomously replicating plasmids the appearance of donor DNA in the recipient's nuclei can be shown at the molecular level (Kellner et al. 1993). The system opens an alternative for sexual recombination over the broad host range of *P. parasitica*. For a wide variety of technical applications it would be sufficient to develop advanced manipulation techniques for a single organism. Plasmid transfer to many recipients could be achieved simply by infection.

6.3 Transformation Systems

Strain improvement by *in vitro* genetic manipulation has met specific problems not encountered in Ascomycota. Most zygomycetes can be transformed with simple plasmid constructs, the only real condition being the availability of a homologous promoter to express the selective marker gene. In this way zygomycetes from the genera *Absidia* (Wöstemeyer et al. 1987), *Mortierella* (Mackenzie et al. 2000), *Mucor* (Van Heeswijck and Roncero 1984), *Parasitella* (Burmester 1992), *Phycomyces* (Revuelta and Jayaram 1986), and *Rhizopus* (Horiuchi et al. 1995) were transformed. Zygomycetes have a strong tendency towards autonomous replication of plasmids. This ability, otherwise unusual in Eumycota may be related to the observation that at least *A. glauca* harbors many different nuclear plasmids (Hänfler et al. 1992). The comparison of artificial vectors for transforming zygomycetes and their ability for replication points at overlaps or even identity between promoter and *ori* elements. In this respect they share common features with Myxomycota. Transformation vectors for mucoralean zygomycetes rely nearly completely on the Tn5-derived gene for aminoglycoside phosphotransferase, mortierellalian species can be transformed due to their higher sensitivity to hygromycin too. Whereas transformation by autonomously replication can be sufficient for analyzing development-specific expression of reporter constructs (Bartsch et al. 2002; Schilde et al. 2001), especially if the vectors carry special elements for stabilizing mitotic segregation (Burmester et al. 1992), this technique does not allow the construction of completely stable derivatives. Therefore, methods for obtaining stable integrative transformants are presently in the center of mucoralean research. Although promising results have been obtained in *A. glauca* by integration via repetitive DNA elements (Burmester et al. 1990), and in *M. circinelloides* (Arnau and Stroman 1993) and *Rhizomucor pusillus* (Wada et al. 1996) after transforming them with *LeuA*-containing plasmids, these approaches had very small efficiencies were far from being reliable or gave rise to severe rearrangements in the introduced DNA. The molecular reasons for the refractory behavior of zygomycetes to integrate foreign DNA remain unclear. The reason does not reside in a general inability to recognize sequence homologies in the genome and in transforming plasmids. After transforming *A. glauca* with plasmids carrying a promoter region from one of the six endogenous genes for elongation factor EF1α, many recombination events were observed at corresponding chromosomal sites, but they were never accompanied by integration of vector DNA (Burmester 1995). The high applicative importance and the additional biotechnical potential of these fungi requires a solution for this pivotal problem in genetic analysis, manipulation, and strain improvement of Zygomycota.

7 CONCLUSIONS

A variety of technical procedures by zygomycetes, between regio- and stereoselective hydroxylation and protease formation, dominates biotechnology with unconventional fungi. Apart from these established processes the biotechnical potential of the basic fungal groups has hardly been scratched. It can be assumed that the enormous biodiversity at the basis of eukaryotic phylogeny, in the noneumycotan groups Oomycota, Myxomycota, and Acrasiomycota as well as in the very ancient divisions of true fungi, Chytridiomycota and Zygomycota, will be the source of novel enzymatic properties, metabolites, and valuable expression systems. The already existing analysis of these organismic groups is very promising at the physiological and at the genetic level.

Established procedures as well as novel ones require genetic analysis, manipulation, and strain improvement. For these purposes, development of efficient *in vitro* tools is indispensable. Modern analytic and predictive tools between analytical genetics, phylogenetics, physiology, bioinformatics, and advanced chip technology will be required in combined efforts to provide us with an adequate access to the word of unconventional fungi. New principles for biotechnology and for fundamental biology are to be discovered especially at the basis of the phylogenetic tree. Together with modern biochip technology a reasonable genetically founded metabolite and property forecast will be within realistic reach. Towards this aim basic biological and genetic research with a variety of organisms will have to be performed on a large scale. We must learn that much more resources and supports

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are needed for high quality science in the forefield of immediate applications if we want to detect novel substances and applications.

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11

Fungal Mitochondria: Genomes, Genetic Elements, and Gene Expression

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1 INTRODUCTION

Mitochondria are the site of vital biosynthetic and degradative metabolic pathways and are responsible for generating most of the energy required for function and survival of eukaryotic cells. Studies of mitochondrial function and biogenesis have traditionally involved many disciplines, including bioenergetic studies of metabolism and oxidative phosphorylation, genetic analysis of inheritance and mitochondrial DNA (mtDNA) mutations, and phylogenetic comparison of mitochondrial genomes. More recently, there has been renewed interest in mitochondria due to the central role they play in programmed cell death (Green and Reed 1998) and the association of mtDNA mutations with several human muscle and neurodegenerative diseases, as well as the aging process (Wallace 1999).

Fungi have played a major role in our current understanding of mitochondria. In particular, the facultative anaerobe *Saccharomyces cerevisiae* has been the favored model organism for genetic and biochemical dissection of mitochondrial function due to the ability to select and recover nonrespiratory mutants. There are numerous reviews covering various aspects of yeast mitochondrial biology (genetics and function; Dujon 1981; Tzagoloff and Dieckmann 1990; morphology and inheritance; Birky 2001; Boldogh et al. 2001; Hermann and Shaw 1998; Yaffe 1999; import mechanisms; Glick and Schatz 1991; Neupert 1997; mtDNA stability; Contamine and Picard 2000; and nuclear-mitochondrial interactions; Poyton and McEwen 1996); however, few reviews focus on nonyeast fungal organisms. The varying range of life styles, life cycles, and environments associated with fungal species demand both flexible and specialized metabolic capacity, and a comprehensive understanding of the role mitochondria play in alternative energy generating pathways will depend on investigations with a variety of fungi. Using the strong foundation established by yeast mitochondrial biologists as a framework, we intend to provide a broad overview of questions that have been or are currently being addressed in nonyeast fungal systems.

2 FUNGAL MITOCHONDRIAL ORIGINS AND FUNCTION

The sequencing of mtDNAs has made it possible to compare the content and organization of entire mitochondrial genomes. In particular, mitochondrial genome sequencing projects carried out by Michael Gray at the Dalhousie University and B. Franz Lang and colleagues at the University of Montreal have provided a number of insightful observations concerning the origin and evolutionary relationships of mitochondria among lower eukaryotes. Aspects of these projects are discussed in chapter 8 with regard to phylogenetic relationships between fungi, yet are worth repeating here as they provide an excellent starting point for understanding the differences in mitochondrial genomes, expression, and nuclear interaction.

Although questions remain about the exact origins of mitochondria, comparative genomic studies provide compelling arguments that support a monophyletic origin and indicate that the ancestor of mitochondria was likely a member of the α-proteobacteria group of eubacteria (Gray et al. 1999; Lang et al. 1999). Significantly, comparisons of phylogenetic trees between mtDNA and nuclear DNA show strong similarities, indicating that the genomes are likely to have evolved in parallel (Gray et al. 2001). The majority of the genes encoded by the ancestral mitochondrial genome were lost, while those involved in bioenergetic functions were retained and most were transferred to the nucleus or were taken over by unrelated

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nuclear genes that perform similar functions (i.e., functional complementation). This seemingly unidirectional transfer process is still occurring, and has been documented in yeast and plants (Adams et al. 2000; Nugent and Palmer 1991; Shafer et al. 1999). These findings suggest that differences in gene content among mitochondrial genomes relate to the degree to which transfers or replacements have occurred.

The sequencing of several eukaryotic nuclear genomes has provided a wealth of information regarding nuclear genes that are involved in mitochondrial biogenesis, maintenance, and function. Structural and comparative genomic studies of the nuclear and mitochondrial genomes of yeast predict that there are at least 423 genes that encode mitochondrial proteins, of which 93% are encoded by the nucleus (Karlberg et al. 2000). Using computational methods, another study estimates that 630, or 10%, of nuclear-encoded genes in yeast are involved in mitochondrial biogenesis and function (Marcotte et al. 2000). A similar number was obtained by analysis of the completed *Caenorhabditis elegans* genome, which suggests there may be some conservation in the overall number between organisms. Interestingly, only slightly more than half of these genes are predicted to have been contributed by the ancestral mitochondrial genome, with the remainder being derived from the host organism itself or other sources. Evidence previously existed for the recruitment of genes from unrelated organisms to complement preexisting mitochondrial gene functions, most notably from T-odd bacteriophages that were the likely source of mitochondrial RNA polymerase. The genomic studies indicate that such recruitments appear to have played a larger role than previously believed and provide a glimpse into the degree to which the ancestral mitochondrion was renovated into the ATP-generating organelle it is today (Karlberg et al. 2000). Studies by Marcotte et al. (2000) also revealed that the percentage of organism-specific mitochondrial genes is very high in yeast (22%) relative to worm (1%), suggesting that mitochondria have different and even specialized functions in different hosts. This may be particularly relevant to fungal organisms, due to the diverse energy sources and metabolic pathways they utilize.

2.1 Fungal Respiratory Pathways

The production of ATP coupled to electron transport is an invariant feature of mitochondria. In animal mitochondria, the respiratory chain begins with electrons being transferred from NADH to complex I (NADH: ubiquinone oxidoreductase) or from the tricarboxylic acid cycle intermediate succinate to complex II (succinate:ubiquinone oxidoreductase). Electrons are transferred via ubiquinones, complex III (ubiquinol: cytochrome *c* oxidoreductase), cytochrome *c*, complex IV (cytochrome *c* oxidase) and finally to molecular oxygen to give water. Although this respiratory pathway is present in most fungal mitochondria, a few fungi, such as *S. cerevisiae* and *Schizosaccharomyces pombe*, lack complex I. More commonly, however, fungi have additional components, such as alternative NADH dehydrogenases and/or an alternative terminal oxidase [reviewed by Joseph-Horne et al. (2001)]. Complex I is a large multisubunit complex that spans the inner mitochondrial membrane and includes both nuclear- and mitochondrial-encoded gene products. In contrast, alternative dehydrogenases are encoded by single nuclear genes and can be located on either the matrix (internal) or intermembrane space (external) side of the inner membrane. Having both internal and external alternative dehydrogenases allow electrons to enter the respiratory chain from either side of the membrane and it is thought that their use may help reduce the production of reactive oxygen species (ROS) generated by the standard respiratory pathway (Joseph-Horne et al. 2001). The alternative oxidase (AOX) is a nuclear-encoded secondary terminal oxidase present in plants and most fungi (*S. cerevisiae* and *S. pombe* are notable exceptions). Although there are differences between fungal and plant AOXs—the fungal AOX is regulated by AMP, ADP, and GDP (Michea-Hamzehpour and Turian 1987; Vanderleyden et al. 1980), while the plant AOX is stimulated by α-keto acids (Rhoads et al. 1998)—they appear to function in a similar manner (Umbach and Siedow 2000). The AOX is located in the inner mitochondrial membrane and catalyzes the reduction of oxygen to water after receiving electrons directly from reduced ubiquinone, circumventing complex III and complex IV. Consequently, cells expressing AOX are insensitive to respiratory inhibitors such as antimycin A and cyanide and ATP production continues due to proton pumping through complex I. In most systems studied, AOXs are subject to multiple forms of regulation and generally operate only under conditions that inhibit standard electron transport. Induction of the AOX could be particularly important for fungal plant pathogens that are exposed to inhibitors of the cytochrome oxidase pathway such as NO released by plants expressing the hypersensitive response (Joseph-Horne et al. 2001). Finally, other studies have found that certain filamentous fungi (e.g. *Fusarium oxysporum*), have the ability to metabolize nitrate to N_2O (denitrification), a process that occurs in the mitochondrion and is coupled to ATP synthesis (Kobayashi et al. 1996). Remarkably, when under anoxic conditions these denitrifying fungi are also able to ferment ammonia (Zhou et al. 2002). Taken together, these studies indicate that fungal mitochondria include several components that are not found in animal cells. The additional components provide greater versatility in aerobic respiration and enable fungi to adjust their metabolic capacity when encountering different energy sources or when in the presence of inhibitors of the cytochrome oxidase pathway.

3 FUNGAL MITOCHONDRIAL GENOMES

3.1 Size and Genetic Exchange

Comparison of mitochondrial genomes among eukaryotic organisms reveals large size differences between animal, fungal, protist, and plant species. The average size of animal

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Table 1 Completely sequenced fungal mitochondrial genomes

Organism	Class[a]	Genome size/ Structure[b]	Acc. #/ Update[c]	Code[d]	Total ORFs[e]	Basic14[f]	Other ORFs[g]	rRNAs/ tRNAs[h]	Reference[i]
Allomyces macrogynus	Chy	57,473 C	NC001715 3/96	U	27	all	*rps3*	2 25	Paquin and Lang 1996
Aspergillus nidulans	Asc-F	33,300[j] C	FMGP	S	~17	all	*rps3* *rnpB*	2 ~22	Brown et al. 1985
Candida albicans	Asc-Y	40,420 C	NC002653 1/01	S	12	all		2 30	Anderson et al. 2001
Harpochytrium #94	Mon	19,473 C	FMGP	U	14	all		2* 8#	FMGP
Harpochytrium #105	Mon	24,570 C	FMGP	U	14	all		2* 8#	FMGP
Hyaloraphidium curvatum	Chy	29,593 L	NC003048 08/01	S	18	all		2* 7	Forget et al. 2002
Hypocrea jeorina (*Trichoderma reesei*)	Asc-F	42,130 C	NC003388 02/02	S	19	all	*rps3*	2 26	Chambergo et al. 2002
Neurospora crassa	Asc-F	64,840 C	Whitehead Institute	S	~30	all	*rps3*	2 27	Griffiths et al. 1995
Pichia canadensis (*Hansela wingei*)	Asc-Y	27,694 C	NC001762 9/95	S	17	all	*rps3*	2 25	Sekito et al. 1995
Podospora anserina	Asc-F	100,300 C	NC001329 01/01	S	50	−atp9	*rps3*	2 27	Cummings et al. 1990
Rhizopus stolonifer	Zyg	54,178 C	FMGP	U	19	all	*rnpB*	2 24	FMGP
Rhizophydium sp. 136	Chy	68,834 C	NC003053 08/01	C	34	all		2 7	FMGP
Saccharomyces cerevisiae	Asc-Y	85,779 C	NC001224 8/99	Y	22	−nadx	*rps3* *rnpB*	2 24	Foury et al. 1998
Schizophyllum commune	Bas	49,704 C	NC003049 08/01	U	20	all	*rps3*	2 24	FMGP
Schizosaccharomyces pombe	Asc-Y	19,431 C	NC001326 11/90	U	10	−nadx	*rnpB*	2 25	Lang et al. 1983
Spizellomyces punctatus	Chy	58,830-C 1,381-C 1,136-C	NC003052, NC003061 NC003060 08/01	C	31	all		2 8#	FMGP
Yarrowia lipolytica	Asc-Y	47,916 C	NC002659 2/01	S	29	all		2 27	Kerscher et al. 2001

[a] Asc = Ascomycete (Y = yeast or non-filamentous, F = filamentous), Bas = Basidiomycete, Chy = Chrytridiales, Mon = Monoblepharidales, Zyg = Zygomycete.
[b] Genome size in bp; C = circular, L = linear.
[c] NCBI accession number or source of information.
[d] Genetic code: U = universal; S = standard mitochondria with UGA coding for Trp, rather than termination; Y = yeast codon usage, with AUA coding for Met, not Ile, CUN = Thr, not Leu, and UGA is Trp, not Ter; C = Chlorophycean mitochondrial code with UAG being Leu, not Ter.
[e] Known polypeptides and ORFs greater than 100 amino acids.
[f] Fourteen genes common to most fungal mtDNAs; *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *atp6*, *atp8*, *atp9*. Minus signs indicate the absence of the gene indicated.
[g] Additional known genes: *rps3* = small ribosomal protein subunit 3 (also includes small ribosomal protein S5 and Var1); *rnpB* = RNA component of RNase P. Intron-encoded proteins are excluded.
[h] Indicates number of rRNA genes (those with asterisk are split) and tRNA genes (those with # contain members that are edited).
[i] FMGP = Fungal Mitochondrial Genome Project (http://megasun.bch.umontreal.ca/People/lang/FMGP/seqprojects.html).
[j] 86% sequenced.

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mtDNA is approximately 16 kb (Boore 1999), whereas plant mtDNAs range from 200 to 2500 kb—larger than some prokaryotic genomes (Palmer 1990). The size of fungal mtDNAs fall between the average animal and lower limit of plant mtDNAs, ranging from 19 kb to 176 kb (Hudspeth 1992). Despite the nearly 10-fold difference in size, there is not a corresponding difference in the number or types of genes encoded by fungal mtDNAs. Most genomes encode the same set of 14 polypeptides, all of which are hydrophobic components of the respiratory chain, as well as a minimal set of tRNAs and rRNAs necessary for their translation (see Table 1). A few additional genes are found in some genomes and all contain several unidentified open reading frames (ORFs). Noncoding regions are usually comprised of AT-rich and repeated sequences as well as introns. Fungal and plant mitochondria also can harbor extrachromosomal DNA plasmids and/or double-stranded RNAs (see below).

A number of factors are likely to have contributed to the size variations in mtDNAs, such as rates of recombination, genetic bottlenecks, and intracellular selection to name a few. Studies of the mechanisms of mitochondrial inheritance, segregation and recombination in model organisms indicate that few generalizations can be made across kingdoms and significant differences are often found between organisms in the same division [reviewed in Birky (2001)]. This particularly applies to fungal organisms. For example, most yeast and other isogametic fungi show biparental inheritance while uniparental inheritance of mitochondria is the general rule for filamentous fungi, although there are many exceptions (Matsumoto and Fukumasa-Nakai 1996; Saville et al. 1998). Several studies have examined the mechanism involved in mitochondrial segregation in *S. cerevisiae* (reviewed by Boldogh et al. 2001; Hermann and Shaw 1998; Yaffe 1999), yet some of the findings may not be highly applicable to other systems due to the uniqueness of the budding process which includes the use of actin filaments for mitochondrial transport versus the use of microtubules in cell division of most other fungi and higher organisms (Brazer et al. 2000; Prokisch et al. 2000). Rates of mtDNA mutation and recombination also vary widely among eukaryotic organisms; plant genomes have extremely high rates of recombination and low rates of mutation (Palmer and Herbon 1988; Wolfe et al. 1987), whereas in animal species the mutation rate is high and the question of whether mitochondrial recombination occurs at all in many genera is still debated (Eyre-Walker 2000). It is generally accepted that the frequency of mtDNA recombination is high in fungi, yet, once again, there are exceptions and it was recently shown that mtDNA recombination in *Candida albicans* occurs infrequently (Anderson et al. 2001).

Filamentous fungi can interact vegetatively via hyphal fusion which provides additional opportunities for genetic exchange and is particularly significant for fungi in which sexual exchange is rare or nonexistent. Anastomosis not only contributes to the variation in mtDNA observed in natural isolates but also plays a major role in the horizontal transmission of mitochondrial mobile elements, such as extrachromosomal plasmids and mobile introns. Surveys of natural fungal isolates indicate that the rate of exchange of mitochondrial plasmids is high (Arganoza et al. 1994) and, in one study, plasmids were even shown to cross vegetative incompatibility barriers (Collins and Saville 1990). Integration of plasmids into mitochondrial genomes generate mtDNA polymorphisms (Griffiths et al. 1995) and, in some cases, can contribute functional sequences to mitochondrial genomes (Nargang et al. 1992). In short, although generalizations concerning the mechanisms of mitochondrial inheritance, segregation, and recombination should be avoided, for most fungi, genetic exchange and recombination between mtDNAs occur at high frequencies. The success of accessory genetic elements, such as plasmids and mobile introns in colonizing fungal mitochondria (see later), reflects this high rate of exchange.

3.2 Sequenced Mitochondrial Genomes

Table 1 contains a list of features associated with 17 mitochondrial genomes that have been completely sequenced and annotated as of April 2002. Twelve of these were obtained from NCBI, while four were obtained from the information posted on the Fungal Mitochondrial Genome Project web page that is maintained by B. Franz Lang at the University of Montreal (http://megasun.bch.umontreal.ca/People/lang/FMGP/seqprojects.html). The sequence for the *Neurospora crassa* mtDNA was obtained from the Whitehead Institute. Annotation of this sequence has yet to be published and the figures shown in the table derive from estimates based on a near-complete (94%) sequence (Griffiths et al. 1995). A hyperlinked version of Table 1 can be found at http://pages.slu.edu/faculty/kennellj/.

The range of fungal organisms listed in Table 1 is quite broad and contains two from Monoblepharidales, four Chytridiales, nine Ascomycetes (5 yeasts, 4 filamentous), one Basidomycete, and one Zygomycete. The large proportion of "lower" fungi on the list derive from efforts of the FMGP to determine their origins and relationship with higher fungi (Paquin et al. 1997). Although there is a five-fold difference in genome size, the genomes show great similarity, particularly in regard to the genes they encode. However, as described below, there are several differences, such as the lack of genes encoding complex I in *S. cerevisiae* and *S. pombe*, number of tRNAs, genome structure and gene order, amount of noncoding regions, introns, and even the genetic code used in translation.

3.3 mtDNA Structure

Fungal mt genomes commonly map as single, circular DNA molecules; however, both linear mt genomes and segmented mtDNAs have been identified. Examples of these are found in Table 1, with *Hyaloraphidium curvatum* having a linear mtDNA and the *Spizellomyces punctatus* genome being composed of three circular molecules. The mt genomes of

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more than 10 fungi, including species of two other genera listed in the table (*Candida* and *Pichia*) have been shown to be linear (Fukuhara et al. 1993; Nosek et al. 1998). There is even evidence to support the idea that linear mtDNAs may represent the major form *in vivo* (Bendich 1996); however, true linear genomes appear to have specific telomeric structures that protect the ends from degradation and ensure that sequence information is not lost during replication (Nosek et al. 1998). For example, the *H. curvatum* genome has telomeres that consist of long (1.4 kb) inverted repeats (Forget et al. 2002) that are similar to structures associated with other fungal linear mtDNAs (Nosek et al. 1998). Finally, the mitochondrial genome of *S. punctatus* is unusual in that it is divided into three circular molecules; a large 58.8 kb molecule and two molecules of approximately 1.2 kb. One of the smaller molecules codes for the *atp9* gene, whereas the other has no identifiable genes (Laforest et al. 1997).

3.4 Gene Content and Genetic Code

Mitochondrial DNAs generally contain genes encoding hydrophobic subunits of respiratory chain complexes, as well as genes for the large and small ribosomal RNAs and a full set of tRNAs (Gray et al. 1999). The standard repertoire of polypeptides encoded by animal mitochondrial genomes includes apocytochrome *b*; cytochrome oxidase subunits 1, 2, and 3; NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5, and 6; and ATPase subunits 6 and 8. Most fungal mitochondria contain the same set of genes plus *atp9* which encodes subunit 9 of the ATPase complex. Interestingly, *atp9* is encoded by both the nuclear and mitochondrial genomes in *N. crassa* and *Aspergillus nidulans* (van den Boogaart et al. 1982; Brown et al. 1985) and is absent from the largest genome (*Podospora anserina*) shown in Table 1. In addition, there are commonly intron-encoded ORFs as well as unidentified ORFs that potentially encode polypeptides greater than 100 amino acids. Many fungal mtDNAs also encode a ribosomal protein associated with the small rRNA. Initially identified in *S. cerevisiae* and called Var1, a second type (*S5*) was also found in *N. crassa*. More recently, a homologue to the bacterial small ribosomal protein 3 subunit (*rps3*) was found in *Allomyces macrogynus*. After re-examination of Var1 and *S5* genes, it was shown they both contain homology to certain regions of *rps3* and are all likely related (Bullerwell et al. 2000). The RNA component of RNase P has also been identified in several mtDNAs (*rnpB* gene, Table 1).

Other notable differences among mtDNAs are the number of tRNA genes. Higher fungi encode a complete complement of tRNAs sufficient to read all codons, based on an extended wobble hypothesis (Bonitz et al. 1980). In contrast, all the members of the Chytridiales included in Table 1, except *A. macrogynus*, have only seven or eight tRNA genes. It is likely that the remainder of tRNAs are nuclear-encoded and are imported into mitochondria (Forget et al. 2002). Most genes are encoded on the same strand and the order of genes within fungal mitochondrial genomes varies widely, a likely consequence of the high rate of mtDNA recombination. One common feature is that tRNA genes are often grouped together and these clusters tend to be dispersed throughout the genome and in some cases are involved in processing transcripts of flanking genes. A third-position codon bias of A and T is commonly observed among fungal mitochondrial genes (Gray et al. 1998), and there are many species that deviate from the universal code (Knight et al. 2001; Nobrega et al. 1980). The stop codon UGA is translated as tryptophan in many Ascomycetes, whereas some lower fungi translate the stop codon UAG as leucine (Paquin et al. 1997). Other deviations are listed in the footnotes of Table 1.

3.5 Introns

Fungal mitochondria contain a large number of introns and they can be divided into two groups, I and II, based on conserved secondary structure features (Michel et al. 1982). Seminal studies concerning the mechanism of self-catalytic and protein-assisted splicing involved fungal mitochondrial introns and many of these have been of great interest due to their ability to move or transpose. There are distinct differences associated with the mobility mechanisms of group I and group II introns although both exhibit a strong preference for the site of transposition, favoring intronless alleles of the gene in which they are associated (reviewed by Belfort and Perlman 1995; Lambowitz and Belfort 1993). This process, called homing, is dependent on intron-encoded proteins that function as endonucleases to cleave the recipient DNA, facilitating DNA transfer (group I) or RNA-mediated transposition involving a reverse transcriptase function of the intron-encoded protein (group II). Comparative genomic analysis of fungal mitochondrial genomes has also made it possible to identify a new class of mobile elements that appear to move by an endonuclease-mediated mechanism similar to group I introns (Paquin et al. 1997). In many cases, the polypeptides associated with mobility also assist in splicing the intron and are classified as maturases. Although most introns have lost the ability to transpose, many retain an association with a maturase protein; introns that do not contain an ORF are usually dependent on proteins encoded by another intron (Henke et al. 1995) or by nuclear genes (Lambowitz and Perlman 1990).

Since homing of mobile group I and II introns is usually site-specific, it is common to find similar introns located at identical positions in widely divergent organisms (Vaughn et al. 1995). They are frequently found in *cox1* and *cob* genes and reside in regions that are highly conserved among species (Paquin et al. 1997). Certain introns are more successful than others, and a recent survey of plant mitochondria indicates that one particularly invasive group I intron has "probably been acquired over 1,000 times separately during angiosperm evolution via a recent wave of cross-species horizontal transfers" (Palmer et al. 2000). Sequencing data indicate that group I introns are more common than group II introns in fungal mtDNAs and their distribution varies greatly.

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For example, no introns have been found in *Schizophyllum commune* or the two closely related species of *Harpochytrium* included in Table 1, whereas 33 group I introns are found in the sequenced A mating type of *P. anserina*. A complete list of organellar introns can be found at: http://wnt.cc.utexas.edu/~ifmr530/introndata/main.htm (Li and Herrin 2002).

3.6 Repetitive Elements

Repetitive sequences are commonly detected in fungal mtDNAs and are frequently associated with recombination events. The elements identified are variable in length and are usually less than 100 bp. Many have conserved secondary structures, such as "G + C clusters" of *Saccharomyces* spp. (de Zamaroczy and Bernardi 1986), "*PstI* palindromes" of *N. crassa* (Yin et al. 1981) and "double-hairpin elements" of *A. macrogynus* (Paquin et al. 2000). The number of repeats within the mtDNA of a particular species varies depending on the strain examined; 81 copies of the double-hairpin element being reported in the 57.5 kb mt genome of *A. macrogynus* and 124 copies of an 11mer repeat in the 100.3 kb genome of *P. anserina* (Koll et al. 1996). The distribution pattern of most of these elements suggests that they are mobile, and for the yeast "G + C clusters" additional evidence for mobility was obtained in crossing studies (Wenzlau and Perlman 1990); however, no mechanism of mobility has conclusively been shown. Regardless, all elements that have been described to date are associated with mtDNA recombination and/or deletion events indicating that they are major contributors to the plasticity of fungal mtDNAs.

4 MITOCHONDRIAL PLASMIDS

In addition to mobile genetic elements associated with mtDNA, extragenomic DNA molecules are frequently detected in fungal mitochondria. These elements can be divided into two groups; those that are derived from the mitochondrial genome via intra- or inter-molecular recombination and true plasmids that are autonomously replicating genetic elements having little or no homology with the host genome. The former group has been intensely studied in the filamentous fungus *P. anserina* where it has been demonstrated that they are associated with a cell death phenomenon, called senescence. Called senDNAs (among other names), the closed circular, multimeric DNA molecules are derived from one of several regions of the mtDNA (reviewed by Griffiths 1992). Juvenile cultures of *P. anserina* are free of the molecules, but one or more types of senDNA is observed in older cultures and the appearance of senDNAs is correlated with a loss of wild-type mtDNA. It is generally assumed that cell death occurs as a result of the loss of mitochondrial function, and the senDNAs appear to accelerate this process (Begel et al. 1999). Recent studies indicate that senescence may be affected by the respiratory status of the cell—cells that are unable to utilize cytochrome oxidase and use the AOX as the terminal electron acceptor escape senescence (Dufour et al. 2000). The authors suggest that longevity may be related to the decrease in the production of ROS formed during normal respiration and the absence of ROS may help maintain the stability of the mtDNA. Other well-studied examples of the formation of circular DNAs derived from mtDNA occur in *N. crassa* (Bertrand et al. 1980; de Vries et al. 1986) and *A. nidulans* (Lazarus et al. 1980). Called "stoppers" or "ragged," respectively, these DNAs are found in certain growth-defective mutants and show similarities to some of the senDNAs.

True plasmids are DNAs (or RNAs) that replicate separately from the mitochondrial genome and usually encode polymerases that are involved in their replication. Initially discovered by chance, surveys of various genera indicate that they are widespread, especially among filamentous fungi. They have been studied most thoroughly in *Neurospora* spp., and it is estimated that more than half of all natural isolates contain one or more plasmid type (Arganoza et al. 1994; Griffiths 1995). The finding of highly similar plasmids in distantly related fungi infers that these elements are horizontally transmitted, and as mentioned previously, this is further supported by laboratory studies that demonstrate plasmids can readily be transmitted via anastomosis.

A list of completely sequenced plasmids is shown in Table 2, which represents less than one third of all reported fungal mitochondrial plasmids. A hyper-text version of this table can be found at http://pages.slu.edu/faculty/kennellj/, as well as an additional list of partially sequenced and/or plasmids identified by hybridization studies. Mitochondrial plasmids fall into specific groups based on their replication cycle, which is dictated by the polymerase(s) they encode. The most common are DNA plasmids (encoding a DNA polymerase), which can be further divided by their structure, being either linear or circular. Linear plasmids have similar features: they usually encode an RNA polymerase in addition to the DNA polymerase and contain terminal inverted repeats having 5′-linked terminal proteins. Circular types generally only have a single ORF encoding the DNA polymerase. Another group of fungal mt plasmids encode a reverse transcriptase (RT), and are classified as retroplasmids. Although similar in size and shape to the DNA plasmids, the replication cycle of the retroplasmids is quite distinct (Kennell et al. 1994). Based on some unique characteristics of the mechanism of replication, it has been proposed that these elements represent a type of "molecular fossil," which is defined as a contemporary genetic element that is ancient in origin (Maizels and Weiner 1993), and thereby can provide information about the evolutionary history of other retroelements, including telomerase (Walther and Kennell 1999; Wang and Lambowitz 1993).

In most cases, mitochondrial plasmids have little or no effect on their fungal host. Although there is a report that at high temperatures they may provide a small benefit to their host (Bok and Griffiths 2000) that could help explain their prevalence, it is generally assumed that plasmids are parasites

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Table 2 Completely and partially sequenced fungal mitochondrial plasmids

Plasmid	Host	Phylum	Size (bp)	Structure	ORFs[a]	Pol.[b]	Acces. #[c]	Update	Reference
pEM	*Agaricus bitorquis*	Basidio	5810	Linear	2	DNA/RNA	X63075	9/99	Robison et al. 1991
pAI2	*Ascobolus immersus*	Asco	5142	Linear	1	DNA	X15982	9/96	Kempken et al. 1989
pClK1	*Claviceps purpurea*	Asco	6752	Linear	2	DNA/RNA	X15648	6/94	Oeser and Tudzynski 1989
pUG1[d]	*Cryphonectria parasitica*	Asco	4182	Circular	1	DNA	Y12637	6/99	Gobbi et al. 1997
pEt2.0L	*Epichloe typhina*	Asco	2000[e]	Linear	(1)	RT	X57200	8/99	Mogen et al. 1991
pFV1	*Flammulina velutipes*	Basidio	8300[e]	Linear	(2)	DNA/RNA	AB028633	9/00	Nakai et al. 2000
pFV2	*Flammulina velutipes*	Basidio	8900[e]	Linear	(2)	DNA/RNA	AB028634	9/00	Nakai et al. 2000
pFOXC1	*Fusarium oxysporum*	Asco	1900[e]	Linear	(1)	RT	AF005240	2/99	Kistler et al. 1997
pFOXC2	*Fusarium oxysporum*	Asco	1905	Linear	1	RT	AF124843	12/99	Walther and Kennell 1999
pFOXC3	*Fusarium oxysporum*	Asco	1836	Linear	1	RT	AF124844	12/99	Walther and Kennell 1999
pFSC1	*Fusarium solani*[f]	Asco	9200[e]	Linear	(1)	DNA	X17602	3/92	Samac and Leong 1988
pFSC2	*Fusarium solani*[f]	Asco	8300[e]	Linear	(1)	DNA	X17603	3/92	Samac and Leong 1988
pG114	*Gelasinospora* spp.	Asco	8231	Linear	2	DNA/RNA	L40494	7/01	Yuewang et al. 1996
pMC3-2	*Morchella conica*	Asco	6044	Linear	2	DNA/RNA	X63909	8/99	Rohe et al. 1991
pHarbin-1	*Neurospora crassa*	Asco	4080	Circular	1	DNA	L42455	3/00	Xu et al. 1999
pHarbin-3	*Neurospora crassa*	Asco	7050	Linear	2	DNA/RNA	NC 000843	4/00	Xu et al. 1999
pMaranhar	*Neurospora crassa*	Asco	7052	Linear	2	DNA/RNA	X55361	7/96	Court and Bertrand 1992
pMauriceville	*Neurospora crassa*	Asco	3581	Circular	1	RT	NC 001570	4/00	Nargang et al. 1984
pFiji	*Neurospora intermedia*	Asco	5268	Circular	1	DNA	L08781	2/96	Li and Nargang 1993
pKalilo	*Neurospora intermedia*	Asco	8642	Linear	2	DNA/RNA	X52106	6/96	Chan et al. 1991
pLaBelle	*Neurospora intermedia*	Asco	4070	Circular	1	DNA	X13912	4/99	Pande et al. 1989
pVarkud	*Neurospora intermedia*	Asco	3675	Circular	1	RT	NC 001571	5/00	Akins et al. 1988
pVS	*Neurospora intermedia*	Asco	881	Circular	0	None	M 32794	8/93	Saville and Collins 1990
pPK2	*Pichia kluyveri*	Asco	7174	Linear	2	DNA/RNA	Y11606	7/99	Blaisonneau et al. 1999
pMLP1	*Pleurotus ostreatus*	Basidio	10,641	Linear	3	DNA/RNA	AF126285	6/99	Kim et al. 2000
pMLP2	*Pleurotus ostreatus*	Basidio	7400[e]	Linear	(1)	DNA	AF355103	4/01	Kim et al. 2000
pAL2-1	*Podospora anserina*	Asco	8395	Linear	2	DNA/RNA	X60707	4/99	Hermanns and Osiewacz 1992
pRS224	*Rhizoctonia solani*	Basidio	4986	Linear	1	RT	AB035862	4/00	Katsura et al. 2001
pThr1	*Trichoderma harzianum*	Asco	2619	Circular	1	RT	AF163325	5/02	Antal et al. 1999[g]

[a] ORFs recognized as encoding polypeptides greater than 100 a.a., numbers in parenthesis indicate predicted polypeptides.
[b] Polymerase: DNA = DNA-dependent DNA polymerase; RNA = DNA-dependent RNA polymerase; RT = reverse transcriptase.
[c] NCBI accession number.
[d] pUG1 has 99% nucleotide sequence identity with pCRY1 (Monteiro-Vitorello et al. 2000).
[e] Partial sequence.
[f] Asexual form of *Nectria haematococca*.
[g] NCBI direct submission: Antal Z, Manczinger L, Kredics L and Ferenczy L, Dept. of Microbiology, Attila Jozsef University, Hungary.

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and represent a type of selfish DNA. In some cases, certain plasmids can be quite detrimental and cause senescence. Two well-studied senescent plasmid groups are linear DNA plasmids of the Kalilo group, and retroplasmids of the Mauriceville group. When strains containing these natural plasmids are subjected to repeated transfer, cultures frequently senesce, a process that is associated with the integration of the plasmid or plasmid-derivatives into the mtDNA and additional recombinations that lead to large deletions or rearrangement in the mtDNA (Griffiths 1992). Other studies show that certain plasmids are also capable of causing mitochondrial dysfunction without integration, by overreplicating and interfering with mitochondrial translation (Stevenson et al. 2000). Although senescence has rarely been observed in nature, these studies indicate that, under certain circumstances, mitochondrial plasmids harm their fungal host. Correspondingly, a mitochondrial plasmid harbored by certain strains of the chestnut-blight fungus *Cryphonectria parasitica* is associated with the attenuation of virulence [reviewed by Bertrand (2000)].

5 MITOCHONDRIAL GENE EXPRESSION

Most of what is known about mitochondrial gene expression has been learned from studies of *S. cerevisiae*. We will briefly summarize the processes of transcription, RNA processing, translation, post-transcriptional modification, and regulatory mechanisms as it occurs in yeast and identify differences that have been reported in other fungal organisms.

5.1 Transcription and RNA Processing

Mitochondrial RNA polymerases are composed of two subunits, a catalytic core subunit (Masters et al. 1987) and a specificity factor (Jang and Jaehning 1991) which appears to function like a bacterial sigma factor. The nuclear-encoded polymerase shows similarity to single-subunit T3/T7-like RNA polymerase and is thought to have replaced the multisubunit bacterial polymerase associated with the ancestral mitochondrial genome (Gray and Lang 1998). The specificity factor binds to the core polymerase creating a holoenzyme that recognizes the mitochondrial promoter. There are at least twelve active promoters in yeast dispersed throughout the genome (eleven on one strand and one on the other; Dieckmann and Staples 1994). They all share the consensus sequence 5′ ATATAAGTA 3′, and the +1 position of transcription is the last A residue (Biswas 1990). All but one primary transcript is multigenic and most contain one or more tRNA. The mitochondrial RNA polymerase of *N. crassa* has been characterized, as have mitochondrial promoters (Chen et al. 1996; Kennell and Lambowitz 1989). The promoter consensus sequence differs slightly from yeast and is not as well conserved among promoters. Most mitochondrial genes appear to be constitutively expressed and differential expression depends on the strength of promoter. Developmental regulation of certain mitochondrial genes has been reported in *N. crassa* during conidial germination; however, regulation was associated with the recruitment of RNAs to the ribosome and not achieved at the level of transcription (Bittner-Eddy et al. 1994). As discussed below, regulation of mitochondrial gene expression predominantly occurs via translational control.

Several processing events are required to generate mRNAs. Most are transcript-specific; however, in yeast, the 3′ end of mRNA precursors are generated by an RNA endonuclease that cleaves RNAs near a conserved dodecamer sequence (Min and Zassenhaus 1993). The 5′ end of tRNAs are cleaved by a mitchondrial RNase P. The RNA component of the ribonucleoprotein complex is encoded in the mtDNA (Miller and Martin 1983) and the protein subunit is encoded in the nucleus and imported (Morales et al. 1992). The 3′ ends of tRNAs are cleaved by a specific endonuclease (Chen and Martin 1988) and CCA residues are added posttranscriptionally by a tRNA nucleotidyltransferase (Chen et al. 1992). Modification of nucleotides of tRNAs appear to occur in the nucleus, cytoplasm and mitochondrion. In the lower fungi, *S. punctatus* and *H. curvatum*, the 5′ end of certain tRNAs are edited (Forget et al. 2002; Laforest et al. 1997) and the pattern of editing is similar to that described in the protist, *Acanthamoeba castellanii* (Lonergan and Gray 1993). As mentioned previously, RNA splicing of group I and II introns has been widely studied and involves intron-encoded maturase proteins as well as nuclear-encoded gene products. A description of these reactions is outside of the scope of this review and we refer interested readers to the following publications for more information: Lambowitz et al. (1999); Saldanha et al. (1993). RNA turnover appears to be important in the regulation of mitochondrial gene expression. Spacer regions cleaved from primary transcripts are rapidly turned over (Margossian and Butow 1996) and enzymes involved in RNA stability have been detected that are transcript-specific (Dieckmann and Staples 1994).

5.2 Translational Control

Reactions involved in transcription and processing may exert some level of control over mitochondrial gene expression, but most regulation occurs during translation. In yeast, 11 of 42 nuclear respiratory-deficient genes (*PET*) affecting synthesis or assembly of mitochondrial gene products are directly involved in translation (Poyton and McEwen 1996) and most, if not all, yeast mitochondrial genes are controlled by inner-membrane-bound translational activator proteins (Fox 1996). These proteins appear to interact with both the ribosomes and the 5′ untranslated regions of mitochondrial mRNAs and help tether the synthesis of hydrophobic mitochondrial gene products to the inner membrane (Green-Willms et al. 1998). Surprisingly, many of the translational activators are mRNA-specific and it is hypothesized that control over individual gene products is needed in order to coordinate the proper assembly of the large respiratory chain complexes.

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Other nuclear genes involved in synthesis and assembly play a role in the import and localization of nuclear-encoded gene products of respiratory complexes. Most of what is known about the mitochondrial import process has been obtained from studies of *S. cerevisiae* and *N. crassa* and an overview of this vital process is discussed in: Glick and Schatz (1991); Neupert (1997); Rehling et al. (2001). Finally, several mitochondrial proteases associated with the inner membrane and matrix have been identified that cleave precursor proteins and a protein having homology to bacterial Lon proteases has been identified in *S. cerevisiae* which is involved in selective protein turnover in the matrix (van Dijl et al. 1998).

5.3 Coordination of Mitochondrial and Nuclear Gene Expression

Mitochondrial function depends on the coordinated expression of genes encoded in the nucleus and mitochondrion. This is evident from the makeup of several mitochondrial complexes, including the translational machinery that involves nuclear-encoded polypeptides and mitochondrially-encoded rRNAs and tRNAs as well as the large respiratory complexes that have subunits encoded in each of the genomes. Initial studies of the regulatory mechanisms that govern nuclear-mitochondrial interactions focused on the expression and import of nuclear genes involved in the respiratory gene complexes (Forsburg and Guarente 1989). In respiring yeast cells, expression of nuclear genes involved in mitochondrial function are under the control of the Hap transcription complex and respond to changes in oxygen level and carbon source (Poyton and McEwen 1996). This complex regulates genes involved in electron transport as well as enzymes involved in the tricarboxylic acid (TCA) cycle and biosynthetic pathways of heme, sterols, and fatty acids. More recently, it has been demonstrated that there is a bi-directional flow of information between the nucleus and mitochondrion and that mitochondria exert some control over nuclear genes (Parikh et al. 1987). Signaling from the mitochondrion to the nucleus, called retrograde regulation, usually involves metabolites as signals and is likely associated with multiple signal transduction pathways (Epstein et al. 2001; Liu and Butow 1999). This form of regulation is triggered by mitochondrial dysfunction and, in yeast, leads to the induction of genes involved in alternative metabolic pathways that appear to maintain vital components of the TCA cycle. The reallocation of resources to the anaplerotic pathways makes sense for yeast, as it is able to survive without mtDNA on fermentable carbon sources. It has yet to be determined whether the same pathways are triggered in obligate aerobes and evidence exists that additional response pathways are present that control genes directly involved in mitochondrial function. For example, the AOX of *N. crassa* is induced by inhibitors of the cytochrome oxidase pathway, as are other nuclear-encoded mitochondrial genes, such as cytochrome *c* (Bertrand and Pittenger 1969; Lambowitz and Slayman 1971; Li et al. 1996). In addition, strains having mutant mtDNAs accumulate defective mitochondria relative to normal mitochondria (Bertrand 1994) and the proliferation of defective mitochondria is analogous to events associated with certain human mitochondrial myopathies (Wallace 1999). These findings suggest that the response pathways in obligate aerobic fungi may have relevance to events associated with human mitochondrial diseases.

6 CONCLUSIONS AND PROSPECTIVE STUDIES

The size and complexity of fungal mtDNAs fall between the relatively small, highly conserved genomes of animals and the highly variable and expansive genomes of plants. There are only slight differences in gene content, with fungal and plant mtDNAs encoding a few additional polypeptides associated with mitochondrial function. Introns, repetitive sequences and noncoding regions all contribute to size variations and most additional ORFs are associated with intron splicing and/or mobility. Although the percentage of noncoding regions is less in fungal mtDNAs than plants, autonomously replicating plasmids are far more common. Fungal mitochondria often contain alternative dehydrogenases and/or an alternative oxidase in addition to the five standard complexes found in animal mitochondria. These alternative respiratory chain components appear to provide increased versatility in aerobic respiration and allow fungi to tolerate compounds that interfere with the normal cytochrome oxidase pathway.

Genome sequencing projects are making it possible to identify the entire complement of nuclear genes involved in mitochondrial biogenesis and function, and are providing new insights into modifications that transformed the ancestral mitochondrion into the energy-producing organelle that exists today. Functional genomic techniques have been used to understand global regulatory changes that occur in response to mitochondrial dysfunction and have shown that metabolic pathways can be reconfigured to maintain optimal energy production. Since mitochondrial gene regulation primarily occurs via translational control, proteomic approaches should be quite informative and may reveal global regulatory mechanisms that directly influence the expression of mitochondrial gene products. It is likely that *S. cerevisiae* will continue to be the pioneering organism in these studies, yet comparative genomic analyses are facilitating the identification and characterization of mitochondrial genes in other systems. The anatomy and developmental diversity of multicellular fungi are ideal for understanding certain aspects of mitochondrial division and developmental regulation, as well as the horizontal transmission of mitochondrial genetic elements. These attributes, among others, will ensure that fungal systems continue to play a major role in our understanding of mitochondrial biogenesis and function in the future.

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12

Fungal Evolution Meets Fungal Genomics

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1 INTRODUCTION

In this contribution, we will review how much is currently known about fungal evolution, and how well phylogenetic hypotheses are supported by the data that were used for these inferences. We will start with the controversial question of how fungi should be classified as an organismal group, using molecular evolutionary criteria, instead of the classical taxonomic and morphological ones, and the extent to which more recent molecular evolutionary results are compatible with the classical views. We will then introduce recent developments in phylogenetics and genomics research that promise to take us another step forward in revolutionizing our understanding of the Fungi, and of eukaryotes in general. By combining information on fungal and other eukaryotic genomes (comparative and evolutionary genomics), genome-wide expression data on mRNAs and structural RNAs (ribonomics or transcriptomics) and proteins (proteomics), and by adding the current knowledge about the interactions of all proteins, RNAs and other molecules (interactomics), fungi have become the best-understood eukaryotic models. In addition, the relatively small genomes of fungi are rapidly deciphered at a moderate cost (e.g., compared to metazoan and plant genomes), and specific genes and gene products are easily analyzed using genetics, reverse genetics, and biochemical techniques that are becoming available for an increasing number of fungi. However, it should be kept in mind that their value as eukaryotic model systems might be limited. Particularly, the intensely studied ascomycete fungi are adapted to rather specialized saprophytic life styles as reflected by their highly reduced gene sets (e.g., compared to animals). It is nonetheless possible that species from other fungal lineages have significantly higher coding capacities that more closely reflect those of other eukaryotes. Through systematic studies of complete fungal genomes from all four fungal phyla we will be able to address this question, as well as further advance the understanding of eukaryotic evolution, of which fungi constitute a substantial portion.

2 A DEFINITION OF FUNGI AND OF THEIR IMPORTANCE

2.1 What Are Fungi?

Early scientists divided eukaryotes into two major groups, Animalia and Plantae. The kingdom Animalia included animals and any other motile organisms that ingest food, including unicellular flagellates and amoebae. Botanists claimed the kingdom Plantae, including plants and other nonmotile organisms that synthesized their own nutrition, i.e., algae. Generally, fungi were also placed together with plants because of their lack of motility, and were considered "lower plants," since they lacked photosynthesis and the development of a root system. However, because of their nonplant-like absorptive nutrition, fungi were regarded as somewhat mysterious plants, and were in some classification schemes included in the kingdom Animalia. As we know today, these classifications do little to reflect the evolutionary relationships of the eukaryotes.

Although mycologists generally agree about what Fungi are, the definition of the term remains fuzzy (e.g., Alexopoulos et al. 1996; Kendrick 2002; Margulis and Schwartz 1988; Margulis et al. 1990; Mozley 2002; Ribes

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et al. 2000). This is mostly due to the influence of traditional classification schemes that continue to remain in some textbooks, and that are based on features that are neither universally valid (like spore-producing eukaryotes with absorptive nutrition), nor characteristic (like cell-wall containing, nonphotosynthetic organisms that are often filamentous, but also occur in nonfilamentous, unicellular forms), nor reflective of evolutionary relationships. Through phylogenetic analysis based on molecular data it has become apparent that organisms previously and currently termed fungi map to most lineages of the eukaryotic tree other than land plants and Metazoa. In turn, numerous species that have traditionally been associated with protists ("protoctists") have recently been identified as Fungi-related based on molecular data (e.g., Microsporidia, Keeling and Doolittle 1996; Keeling and McFadden 1998; Pneumocystis carinii, Edman et al. 1988; Hyaloraphidium curvatum, Forget et al. 2002; Ustinova et al. 2000).

Although many authors discussing fungal taxonomy have been critical about the lack of scientific standards in defining their subject, some have attempted to remain diplomatic at the same time. In one of the most recent standard mycology textbooks (Alexopoulos et al. 1996), the authors state in the introduction: "*To define the exact limits of the group is very difficult . . . . In this textbook, we will take a very broad approach and will discuss all major groups of organisms that*

Table 1 Systematics of Fungi and fungus-like eukaryotes

Kingdom Fungi ("true fungi", "eufungi")	
Phylum Chytridiomycota	
Blastocladiales	(*Allomyces* spp.; *Blastocladiella emersonii*)
Spizellomycetales	(*Spizellomyces* spp.; *Rhizophlyctis rosea*)
Monoblepharidales	(*Monoblepharella* spp.; *Hyaloraphidium curvatum*)
Chytridiales	(*Rhizophydium* spp.; *Chytridium convervae*)
Neocallimasticales	(*Neocallimastix* spp.)
Phylum Zygomycota[a]	
Trichomycetes	
Harpellales	(*Smittium* spp.)
Zygomycetes	
Dimargaritales	
Endogonales	
Entomophtorales[b]	
Glomales	
Kickxellales	
Mucorales	(*Rhizopus* spp.; *Mucor* spp.; *Mortierella* spp.)
Zoopagales	
Phylum Basidiomycota	
Hymenomycetes	(*Schizophyllum commune*; *Cantharellus cibarius*)
Urediniomycetes	
Ustilaginomycetes	(*Ustilago* spp.)
Phylum Ascomycota	
Euascomycota (Pezizomycotina)	(*Neurospora* spp.; *Aspergillus* spp.; *Penicillium* spp.)
Hemiascomycota (Saccharomycotina)	(*Saccharomyces cerevisiae*)
Archiascomycota (Taphrinomycotina)	(*Schizosaccharomyes* spp.?; *Taphrina* spp.; *Saitoella* sp.?)
Kingdom Stramenopila (heterokonts; "pseudofungi")[c]	
Oomycota	(*Phytophthora infestans*)
Hyphochytriomycota	(*Rhizidiomyces* spp.; *Hyphochtrium* spp.)
Labyrinthulomycota	(*Labyrinthula* spp.)
Kingdom Amoebozoa ("pseudofungi")[c]	
Mycetozoa	(*Dictyostelium* spp.; *Physarum* spp.)

Examples of representative species are indicated in brackets; classifications are based on the GenBank/NCBI taxonomy, slightly modified to reflect more recent phylogenetic results and definitions.

[a] One of the Entomophtorales, *B. ranarum*, is possibly a chytridiomycete (Jensen et al. 1998).

[b] Subdivisions based on uncertain phylogenetic inferences (e.g., Tanabe et al., 2000).

[c] Incomplete listing of subgroups.

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have been called fungi over the years as well as other organisms, such as the slime molds, that historically have been studied by mycologists." The same authors then go on to give a more restrictive definition of the kingdom *Fungi*, an assembly of organisms including Chytridiomycota, Zygomycota, Basidiomycota, and Ascomycota, justified by the monophyly of these groups in phylogenetic analyses based on molecular data. In contrast, in two other distinguished textbooks (Margulis and Schwartz 1988; Margulis et al. 1990), fungi are defined as "*haploid or dikaryotic organisms developing from desiccation-tolerant spores and lacking undulipodia at all stages of their life cycle,*" thus excluding the Chytridiomycota (or "chytrids") from the Fungi, and in turn associating chytrids with "Protoctists", an immense group of most diverse eukaryotic organisms defined by exclusion of other eukaryotes: not-plant, not-animal and not-fungus. Obviously, this negative definition does little to help define, in scientific terms, how fungi are best distinguished from other eukaryotes, and neglects that Chytridiomycota are phylogenetically related, and therefore best included in, the "*true fungi*."

Some authors have taken the direct approach of naming all nontrue-fungal (in a phylogenetic sense) organisms "*pseudo-fungi*" (Cavalier-Smith 1987), a term that has not been well-received among mycologists; others (Bruns et al. 1992) have proposed the use of the term "*fungi*" in the traditional sense, and the term "*Fungi*" (with a capital "F") for fungi in a scientifically defined (phylogenetic) meaning, as defined in Alexopoulos et al. (1996).

To avoid possible confusion due to the difference between the traditional and scientific meaning of the term, in this review, we will use the term "Fungi" ("fungi") only in a phylogenetic sense. More specifically, because it is now generally accepted that Fungi and Metazoa diverged from a common eukaryotic ancestor, Fungi are defined as the group of organisms forming a sister clade of the Metazoa, including Chytridiomycota, Zygomycota, Basidiomycota, and Ascomycota (Table 1).

The proposed molecular taxonomy system of classification implies that it be constantly updated following new phylogenetic results, and by employing statistical criteria to ensure association of species or groups of organisms within a given phylogenetic network, with significant support. Although at first this approach might appear straightforward in a scientific sense, it has its limitations when it comes to defining "significant support" for given tree topologies (see Section "Tree Building Techniques"). According to our analyses, much genome sequencing and phylogenetic analysis is still required to meet the ultimate goal of a scientifically sound classification scheme of the Fungi.

2.2 Why Should We Study Fungi?

While there is little doubt that fungi are "important," as outlined below (and to which the many contributions to this book testify), applied research and technology development is only one facet of scientific progress. In fact, applied research critically depends upon (and is indeed fueled by) scientific results that have been realized through studies of often economically irrelevant model species, emerging from curiosity-driven science.

Fungi have always played an important role in human life. For instance, mushrooms have been collected in the wild since the beginning of mankind and are now cultivated in industrial quantities (an industry of close to US$ 1 billion/year worldwide); for several millennia, yeasts have been used to prepare bread and to ferment wine and beer, and various yeast strains are now subject to improvement by large industries; *Aspergillus* is employed for soy sauce fermentation, as well as the industrial production of numerous enzymes and organic compounds, etc. While fungi have the potential to be employed in a much greater production of organic compounds and enzymes, we know little about the genetics, genomics, and biochemistry of these economically relevant species. In fact, most strain selection and strain improvements are driven by trial and error, rather than by scientifically rational design.

Fungi are also an essential component of our ecosystem. There are numerous interactions between fungi and plants; in fact, the growth of most trees (and consequently the existence of forests and by implication a thriving forest industry) critically depends on efficient mycorrhizal symbiosis. Animals also benefit from associations with fungi. Symbioses between free-living fungi and ants have been described (Currie 2001), as have those between yeastlike endosymbionts and certain insect species (Suh et al. 2001).

While the list of beneficial properties of fungi is much too long to fully detail in this review, so is the number of diseases caused by them. Certain mushrooms produce toxins that may prove fatal if ingested (e.g., *Amanita phalloides*: "death cap"). Others (of the genus *Psilocybe*) affect the central nervous system, inducing hallucinogenic responses. Many molds produce secondary metabolites (mycotoxins) that are highly toxic or carcinogenic, such as flavotoxin from the euascomycete *Aspergillus flavus* (Etzel 2002). Ergotism, also refered to as "Saint Anthony's Fire," is caused by the ingestion of bread prepared from rye infected with the fungus *Claviceps purpurea*. Historically, several outbreaks of widespread mental illness have been attributed to ergotism, and have provoked mass persecution of afflicted individuals (e.g., in the witch trials of Salem, Massachusetts).

Finally, a multitude of plants are attacked by fungi, causing considerable economic loss in forestry (e.g., *Armillaria*) and agriculture. An estimated 10% of the world's food supply is lost to various ascomycetes and basidiomycetes; e.g., through *Cochliobolos heterostrophus* corn leaf blight, *Magnaporthe grisea* rice blast, a variety of plant diseases caused by *Fusarium* spp. and *Rhizoctonia solani*, etc. The economically important light blight disease of potatoes is caused by the oomycete *Phytophthora infestans*,

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a filamentous growing protist often referred to as a fungus, which actually belongs to the diverse group of stramenopile protists.

3 WHY USE GENOMICS AND EVOLUTIONARY APPROACHES

3.1 Why Investigate Fungal Genomes and Gene Expression at the Genome Level?

Despite the scientific and economic importance of a wide variety of fungi, only a few species, almost exclusively Ascomycota, are widely used in molecular and genetic studies. In particular, the budding yeast, *Saccharomyces cerevisiae*, the fission yeast, *Schizosaccharomyces pombe*, and the two filamentous euascomycetes *Aspergillus nidulans*, and *Neurospora crassa* have been extensively studied to unravel principles of genetics, gene regulation, and gene expression. The nuclear and mitochondrial genomes of all four species have been sequenced, or are near completion. From a historical standpoint, the reasons for this biased choice of species as eukaryotic model organisms are obvious and compelling, including the ease of propagating cultures on a variety of defined synthetic or semi-synthetic media, the potential of growing large quantities of cells for biochemical studies, well-developed classical as well as reverse molecular genetics, and the possibility of strain preservation, either cryogenically or in dehydrated form.

Saccharomyces cerevisiae was first studied at the genetic level in 1935 (Winge 1935). Since then, intense biochemical and genetic studies have created a body of scientific knowledge rivaling even that of *Escherichia coli*. In fact, *S. cerevisiae* is generally seen as the *E. coli* of eukaryotes. The complete genome sequence of *S. cerevisiae* was published in 1996 (Goffeau et al. 1996) and several databases that integrate the rapidly growing information on this organism have been created (e.g., Saccharomyces Genome Database, http://genome-www.stanford.edu/Saccharomyces/; MIPS Yeast Genome Database, http://mips.gsf.de/proj/yeast/CYGD/db/). The availability of a complete eukaryotic genome sequence well before those of other eukaryotes reached completion, combined with the existence of a vibrant community of yeast researchers, has led to the development of several revolutionary technologies for the investigation of *S. cerevisiae* that are to some degree also applicable to other eukaryotes. Deviating from the focus of traditional biochemistry and molecular biology on single genes and gene products, these new technologies address issues at the genomic level. Examples of such "big questions" include the systematic, genome-wide identification of protein interactions using the yeast two-hybrid system (e.g., Uetz and Hughes 2000), or alternatively, large-scale protein sequencing of protein complexes by mass spectrometry (e.g., Ho et al. 2002; Gavin et al. 2002), the genome-wide location of DNA binding proteins (Ren et al. 2000), and the analysis of protein activities using protein chips (Zhu et al. 2001). While any of these new techniques has its specific limitations, the combined evaluation of different genomics data sets provides information that compensates for many of these shortcomings (e.g., Ge et al. 2001; Jansen et al. 2002).

From what is outlined above, it is undeniable that the budding yeast *S. cerevisiae* has become the organism of choice for the development of new genome methodologies for eukaryotes. However, as a model system, budding yeast has some limitations. There is virtually no other eukaryote with such easily applied genetics and reverse genetics techniques, thus using these methods elsewhere is easier said than done. In addition, budding yeast has a tiny nuclear genome compared to most animals and plants, containing a small fraction of the genes encoded by the genomes of most of its eukaryotic cousins. Could there be fungal model systems that better represent the more complex eukaryotes? An alternative choice of fungal model organism is the fission yeast *S. pombe*, a member of the Archiascomycota (Taphrinomycotina, according to the NCBI taxonomy, see Table 1), an excellent complement to budding yeast in studying areas such as cell cycle and cell division. The publication of the complete *S. pombe* sequence (Wood et al. 2002) reveals that it has the smallest number of protein-coding genes recorded so far for a free-living eukaryote, although, compared to *S. cerevisiae*, there is little difference in the basic set of genes. Budding yeast has many duplicate genes whereas fission yeast does not, which explains why *S. pombe* has such a low total gene count while retaining a similar set of cellular functions. The lack of genome duplication makes *S. pombe* a potentially superior (less complicated) model system than *S. cerevisiae*. In addition, nucleus-encoded *S. pombe* genes and proteins have long been recognized to be more similar to their mammalian homologs than they are to their *Saccharomyces* counterparts. This feature inspired many molecular biologists to believe that *S. pombe* might be more closely related (in evolutionary terms) to animals than to *S. cerevisiae*, a conclusion that is unfounded. Both "yeasts" belong to the "higher," ascomycete fungi as confirmed by virtually all taxonomic classifications and molecular phylogenetic analyses. However, while budding yeast has undergone a largely accelerated evolution of both its nuclear and mitochondrial genes, the nuclear genome of fission yeast has evolved at a much lower rate. This makes nuclear gene sequences of fission yeast more similar to those of animals and all other eukaryotes, which is often a decisive advantage in molecular comparative analyses. However, it does not justify the conclusion of Sipiczki (1995) that fission and budding yeasts are as different from each other as either is from animals (see phylogenetic distances and tree topologies in Figures 2, 3 and 4). On the other hand, fission yeasts and budding yeasts belong to distant lineages within the Ascomycota and are possibly more distantly related than either *S. pombe* or *S. cerevisiae* is to the euascomycetes (Pezizomycotina), *N. crassa*, or *A. nidulans* (see the discussion of phylogenetic relationships).

Finally, other fungal model organisms that merit mention are the filamentous euascomycetes *N. crassa* and *A. nidulans*

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(the latter species is also known as *Emericella nidulans*, according to a more recent but not well-accepted nomenclature proposal), which have been intensely studied genetically and biochemically over several decades, rivaling to some degree even *S. pombe*. There are several research groups that contribute to genome projects in these organisms (e.g., http://www.genome.ou.edu/fungal.html; http://biology.unm.edu/biology/ngp/home.html). It is to be expected that the genomes of these two fungi harbor many more genes than their derived yeast cousins, due to the presence of additional metabolic and cellular functions. Other fungal genome projects target the budding yeast *Candida albicans* and the basidiomycete *Schizophyllum commune* (http://genome.semo.edu/). Finally, the "Fungal Genome Initiative" proposed the sequencing of 15 fungal genomes relevant to the field of medecine, agriculture, industry, comparative genomics, and evolution. Depending on available sequencing capacity, the Genome Resources and Sequencing Priority Panel (GRASPP) at the National Human Genome Research Institute (http://www.genome.gov) suggested sequencing genomes of only 4 ascomycete and 3 basidiomycete species (*Cryptococcus neoformans* Serotype A, *Pneumocystis carinii*, *Magnaporthe grisea*, *A. nidulans*, *Fusarium graminearum*, *Coprinus cinereus* and *Ustilago maydis*).

It is worth noting that basidiomycetes and lower fungi (zygomycetes and chytridiomycetes) have not been developed as models to the same degree as the ascomycetes. For instance, the chytridiomycete *Allomyces macrogynus* was used as the main lower fungal model for a certain period (e.g., Olson 1984), but researchers have virtually abandoned working on this species in the last two decades. Indeed, to our knowledge there are currently no ongoing nuclear genome sequencing projects of nonascomycete/basidiomycete fungi. Clearly, we have not even begun to address the question of what Fungi are, in terms of genomics, in a systematic manner.

3.2 Reasons to Apply Evolutionary Approaches

The understanding of evolutionary relationships is essential for two reasons other than curiosity about fungal evolution itself: (a) the importance of fungi for economic or health-related issues, e.g., in order to put them to use in the most efficient way, or to treat diseases caused by them. One apparent example is in the treatment of *P. carinii* infections: knowing that it is an ascomycete fungus and not some ill-defined protist, it is easier to identify and to develop appropriate treatments (Edman et al. 1988). Another example is the infection of plants by oomycetes (e.g., *P. infestans* in potatoes); again, knowing that it is *not* a fungus but a member of the Stramenopila provides important leads for the development of treatments in combating this plant pathogen; (b) use of evolutionary principles to understand the structure and function of macromolecules (proteins, structured RNAs), in connection with sequence variation and gene evolution (comparative phylogenetic modeling). This last category of applications can be thought of as a complement to biochemical and molecular analyses in the understanding of molecular structure and function, a principle that is increasingly applied in genome-wide studies of newly sequenced model organisms.

4 EVOLUTIONARY CONCEPTS AND TECHNIQUES

4.1 What is Phylogenetics?

Phylogenetics is part of a science called systematics. The latter is an attempt to understand the evolutionary relationships of living organisms, in order to interpret the way in which life has diversified and changed over time. While classification is primarily the creation of names and groups, systematics goes beyond to elucidate new theories of the mechanisms of evolution. Phylogenetic systematics is the method used to reconstruct the patterns that have led to the distribution and diversity of life, and phylogenetic studies are no longer limited to biological classification, but have permeated almost every subdiscipline in biology: comparative biologists appreciate the importance of phylogenetic methods for interpreting and predicting all kinds of biological patterns and processes. The core concept of phylogenetic systematics is the use of synapomorphic characters (for an explanation of evolutionary terms, see the glossary) to reconstruct common ancestry relationships and grouping of taxa based on common ancestry. This concept has gradually revolutionized the nature of traditional systematics, in which scientists select the morphological character(s) believed to be important, and delimit and group species based on these characters. However, due to the subjectivity of the traditional approach, it is often difficult to evaluate the evolutionary significance of such taxonomic groups as they may be defined by characters that have little or no evolutionary relevance (e.g., filamentous growth).

4.2 The Inference of Phylogenies from Sequence Data

As mentioned previously, phylogenetic methods use common attributes and shared characters to trace the evolutionary history of living organisms. These characters can be morphological or molecular (sequence, restriction endonuclease patterns, isozyme patterns, gene order, etc.). In this review, we will limit ourselves to the discussion of phylogenetic concepts related to sequence data, because it has become the most powerful and widely used approach.

Evolutionary biologists shudder when the terms similarity and homology are used indiscriminately. Sequence similarity measures the degree of identity without reflecting the causality of resemblance, which may result from chance, convergence, but which is not necessarily based on a shared origin. Homology refers to a relationship by descent (common ancestry). Evolutionary relationships are often inferred by

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simply comparing similarities (e.g., BLAST scores) of gene homologs among various species. However, the observation that some *S. pombe* genes are more similar to animal genes than to *S. cerevisiae* does little to help elucidate the evolutionary history of these genes or the organismal groups in which they reside. Sequence similarities correlate with the rate of evolution in a given organismal group, and in our example, the high similarity between *S. pombe* and animal sequences only indicates that *S. pombe* evolves at a lower rate than *S. cerevisiae*. Evolutionary relationships can (and should) be inferred with algorithms that account for variable rates of evolution, that assess the stability (robustness) of inferred topologies, and that can be represented by branching patterns (phylogenetic trees). In order to construct a phylogenetic tree one must first align sequences (nucleotide or protein) that are sufficiently similar to permit unambiguous alignment of at least part of the sequences, extract unambigiously aligned sequence blocks, apply tree inference methods, and finally evaluate the validity of the tree. One should keep in mind that a phylogenetic tree is and remains a hypothesis, independent of the degree of statistical support.

4.3 Species Choice

The first question that faces scientists in the construction of phylogenetic trees is which and how many species should be included. In theory, the highest possible number of species that are evenly distributed over the tree is ideal (Lanyon 1985; Lecointre et al. 1993). For example, to build a global fungal phylogenetic tree, sequences from three or more taxa for each major clade should be included, i.e., at least 30 well-selected species. While this method sounds simple enough, lack of data from the lower fungal phyla (Chytridiomycota, Zygomycota) becomes a major obstacle in its application.

Although, in principle, the inclusion of large numbers of species produces a more robust phylogenetic tree, there are other reasons that may justify the elimination of species from a data set: (a) the reduction of computation time; and (b) the stabilization of the overall topology when species are found to have long branches (i.e., highly derived sequences that introduce more "noise" than phylogenetic signal). The tendency of long phylogenetic branches to attract one another strongly disturbs tree inferences (Hendy and Penny 1989), and might lead to the prediction of relationships that are highly supported in statistical tests but are nonetheless incorrect.

The choice of an outgroup is another important issue when species are selected. Species in the outgroup should not be so far from the ingroup (those species for which a phylogenetic tree is sought) that alignment becomes problematic; however, there must be no doubt that the outgroup taxa are indeed outside of the group under study. The inclusion of multiple taxa in the outgroup helps to increase the confidence level of the resulting tree, and might be useful for the reduction of long-branch attraction artifacts (Smith et al. 1994). In addition, multiple successive outgroups can provide a test of ingroup monophyly. For example, to study fungal global phylogeny, animals plus recent ancestors of both animals and fungi generally make a suitable outgroup.

4.4 Data Set Choice

Ideally, sequence data suitable for phylogenetic inference is relatively well conserved for resolving long distances and less conserved for closer relationships. For a given protein coding gene, DNA sequences have three times as much data as protein alignments, but generally do not provide more phylogenetic signal because of noise introduced by the more variable first and particularly third codon postitions, due to redundance of the genetic code. Additional noise is incorporated when organisms have alternate codon usage (e.g., the specification of UGA for tryptophan in various fungal mtDNAs), or when the nucleotide composition is biased and highly variable (e.g., most mitochondrial sequences are A + T rich, and the degree of A + T bias varies over a wide range). As a result, DNA is most appropriate for the resolution of close phylogenetic relationships, while amino acid sequence data is more appropriate for distant organisms, essentially because of the highly conservative nature of protein evolution (Swofford et al. 1996).

The question of data quantity is not so easily answered. Longer sequences are less biased by random noise, which has led many scientists to concatenate sequence data from different proteins or genes. However, one should be aware that concatenated sequences must be of the same type (i.e., amino acid sequence cannot be concatenated with ribosomal RNA data), and should display a similar rate and mechanism of evolution (which is questionable for a mix of rRNA and nucleotide sequence of protein-coding genes, for instance). Sequence concatenation, of course, depends on data availability, and the analysis of longer sequences is also more computationally intensive.

When choosing genes for phylogenetic studies, horizontally transferred genes should be avoided since these do not reflect the history of the host genome. In eukaryotes, this problem is not as widespread as in prokaryotes. However, eukaryotic genomes are often affected by gene duplications, and care should be taken to consider alternative scenarios of gene loss vs. gene duplication in the interpretation, or otherwise avoid data from gene families if possible.

4.5 Sequence Alignment

In addition to using sequences from homologous molecules, phylogenetic inference from sequence data requires positional homology, i.e., sequence alignment, which is among the most difficult and subjective components of phylogenetic analysis. It is quite common that the alignment is not produced by a dedicated software alone, but that the user applies a final manual "refinement" that can to some degree be justified by

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the imperfection of available alignment software. Another justified, but similarly subjective decision is the elimination of regions with uncertain positional sequence similarity. If not removed from the data set, these sequences will introduce "noise," masking the phylogenetic signal.

4.6 Tree Building Techniques

Several methods can (and should) be used for comparative purposes, each with its specific limitations and advantages. The majority of these methods fit into three main groups: parsimony, distance, and maximum likelihood. Parsimony methods select for trees that minimize the total tree length (the number of evolutionary steps required to explain a given set of data). This method is appropriate for the analysis of morphological data, and a highly developed suite of programs for parsimony analysis is available for various computing platforms (PAUP*, Swofford 1996). However, parsimony has been shown to be inconsistent when applied to molecular sequence data (Felsenstein 1978; Kim 1996), and is highly sensitive to bias within the data (e.g., unequal amino acid frequencies, amongsite rate variation, amongtaxa amino acid composition differences, longbranch attraction).

Maximum likelihood methods optimize the statistical likelihood for a tree based on a data set, and given a specified evolutionary model of character changes. These methods are best suited for the analysis of molecular data as they are unmatched when dealing with bias, noise, and error. Modern phylogenetic analysis packages incorporating maximum likelihood often provide a variety of amino acid substitution models that account for unequal amino acid frequencies and variability of substitution rates across sites. The major drawback to the use of maximum likelihood is its high demand for computational resources.

Phylogenetic inference by the much faster distance methods is a two-step process. The sequence data are first converted into a set of discrete pairwise distance values arranged in the form of a matrix. The phylogenetic tree is then constructed by cluster analysis methods (e.g., neighbour-joining and variants thereof, or minimum evolution methods). These methods are only mildly susceptible to bias within the data and can be corrected by amino acid or nucleotide substitution models. The main drawback is that tree topologies are not constructed directly from sequence data but *via* an intermediate distance table, which results in loss of phylogenetic signal, especially when long concatenated sequences are used.

4.7 Evaluation of the Tree

As phylogenetic trees are evolutionary hypotheses that critically depend on the amount and quality of data that was used to build them, these hypotheses should always be tested. The simplest and most widely used tests are based on data resampling from the original data set (namely Bootstrap and Jackknife). Bootstrapping consists of random drawing of columns from a sequence alignment, as many times as there are columns. The result is a data set of the original data set size, from which some aligned positions have been eliminated, while others are present in multiple copies. The Jackknife technique involves the random deletion of a specified fraction of alignment columns, producing a data set that is smaller than the original (e.g., deletion of 50% of sites produces a data set half the size of the original). Using one of these methods, a large number of data sets (at least 100, usually 1000) are produced, and trees are inferred from each. A consensus tree is produced and percentage values are calculated for each internal branch, indicating the number of resampling topologies in which it was found. For support to be considered significant, the resampled data sets should frequently predict the same branches. To the great surprise of many statisticians and biologists who are used to applying a standard p-value of 0.05 (or 95% support), there is little consensus in the community of phylogeneticists on the meaning of "significant" Bootstrap or Jackknife support; some authors consider values above 50% to indicate support, some go even below this value. We, together with many others, insist that only values above 95%, or at least 90%, indicate significant support, although even at this level there is no guarantee that the tree topology will not change as more sequence data and additional species are added. Another reason to be fairly critical even of high support values is that resampling techniques are not rigorous statistical tests that would take into account overall support of a given tree topology, and can therefore be positively misleading (e.g., see the well-documented example in Shimodaira 2002).

More reliable, but computationally demanding alternatives are likelihood tests, which evaluate the statistical probability of competing tree topologies. Given several possible topologies, these tests return confidence values that indicate whether the most probable tree topology is significantly better supported than its alternatives [e.g., Kishino and Hasegawa (1989); Shimodaira (1999); Shimodaira and Hasegawa (2001); Shimodaira 2002; Yang (1997); see also the excellent review on molecular phylogenetics by Whelan et al., (2001)]. Likelihood tests have not been popular among users of phylogenetic approaches. These tests are often considered too rigid as they deny support for a major fraction of branches of most published trees, at a standard confidence value of 0.05, and especially when deep divergences based on only single gene sequences are analyzed. There has been also substantial confusion about the use of the available tests. The widely used Kishino–Hasegawa (KH) test (Kishino and Hasegawa 1989) is not applicable for the comparison of trees that have been selected *a priori*, so should not be applied for testing a favorite tree topology against a chosen number of alternatives. For such questions, the Shimodaira–Hasegawa (SH) test (Shimodaira 2001) can be applied, although it is excessively critical in assessing the significance of likelihood differences. Support by the SH test strongly decreases as an increasing number of tree topologies is tested, a difficulty that is partially eliminated in the weighted SH test (wSH;

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Shimodaira 2002). The less severe, least biased, and currently most appropriate testing method appears to be the recently developed "Approximately Unbiased" (AU) test (Shimodaira and Hasegawa 2001; Shimodaira 2002). In our experience, the AU test performs adequately to avoid overconfidence in phylogenetic hypotheses, without rejecting all but the most evident branching orders.

4.8 The Evolution of Phylogenetic Methods

The rapid progress in the development of phylogenetic inference methods provokes questions concerning the validity of previously published phylogenetic results, especially when they are based on only single gene or protein sequences that tend to provide little statistical support, at large phylogenetic distance. In addition, discoveries of evolutionary patterns that contradict the assumptions of current treebuilding algorithms, such as covariation of sequence change (Lockhart et al. 1992; Lockhart et al. 1998; Philippe and Lopez 2001) have yet to be adequately implemented in tree building algorithms. One must also consider the possibility that, in some cases, the phylogenetic signal might be so weak that deep branching patterns only reflect the combined effects of various systematic biases rather than true phylogenetic relationships (Forterre and Philippe 1999; Lockhart et al. 1998; Lopez et al. 1999; Philippe and Laurent 1998).

How reliable, then, are gene-sequence-based phylogenies in describing organismal evolution? How serious are discrepancies due to methodological errors? To what extent do molecular data contain sufficient traces of their ancient past to correctly infer fungal phylogeny? These critical questions are no reason for a general skepticism about the validity of molecular phylogenetic inferences. It is likely that sufficient phylogenetic signal for the resolution of divergences as deep as the first eukaryotes, or even the first living cellular organisms, can be found in the complete genome sequences of extant species. This level of inference calls for sequencing of a large number of complete genomes across a broad spectrum of species.

Table 2A Likelihood tests of tree topologies. rRNA data of Nishida and Sugiyama (1993) vs. ML topology (not shown)

Tree topology	Dli	AU	wSH	NP
(a) ML topology (best; our analysis)	−20.6	**0.976**	**0.975**	**0.974**
(b) topology of Nishida and Sugiyama	20.6	0.024	0.025	0.026

Statistical tests to evaluate relative likelihood of topology published by Nishida and Sugiyama (1993), compared to our maximum likelihood analysis of the same data set. The values represent, from left to right, the likelihood distance (Dli), the p-value of the Approximately Unbiased test (AU), the weighted Shimodaira–Hasegawa test (wSH), and the bootstrap probability of selection (NP). All tests were perfomed as described in Shimodaira (2002) applying an alpha factor of 0.25. There are several topological differences between these two trees. In the maximum likelihood tree, the Fungi are monophyletic, whereas in the Nishida and Sugiyama tree, the zygomycete and chytridiomycete included in the analysis form a monophyletic group with the oomycetes. In addition, the group formed by the archiascomycetes branches prior to the split between the Ascomycota and the Basidiomycota in the maximum likelihood tree, while this group is at the base of the Ascomycota in the tree published by Nishida and Sugiyama. Despite this clearly odd feature of our maximum likelihood analysis, the Nishida and Sugiyama tree has p-values well below the confidence level (0.05), and can therefore be rejected.

5 CURRENT KNOWLEDGE ON FUNGAL EVOLUTION

5.1 Overview

In this chapter, we will summarize current results of phylogenetic inferences with a few selected data sets, discuss the consistency among these results, and their bearing on fungal taxonomy. Although morphological characters have successfully been used in the past both to classify organisms and to infer phylogenies, the progression towards molecular data as the standard basis for phylogenetic inference is particularly critical in the case of the Fungi, as many morphological characters historically deemed most useful for the study of phylogenetics are convergent, reduced, or missing (e.g., mechanisms of sexual reproduction). The use of molecular data in fungal phylogenetics has allowed the placement of asexual fungi near the organisms to which they are most closely related, something that was impossible with the morphological characters of sexual reproduction traditionally used in fungal classification. Molecular data show that the Microsporidia, once thought to be ancestral protists (Leipe et al.,1993), are, in fact, highly derived Fungi (Keeling and McFadden 1998), whereas other groups previously classified as Fungi (e.g., oomycetes, hyphochytriomycetes, and slime molds) have been excluded (Bhattacharya et al. 1991; Van der Auwera et al. 1995).

The first molecular phylogenies of the Fungi were based on rRNA sequence. Now, as an increasing number of sequences from nuclear and mitochondrial protein-coding genes are determined, it is possible to use alternative molecular data sets to infer fungal phylogenies. As more data are produced, the use of concatenated sequence data is becoming a possibility. The rationale for using concatenated sequences is that: (a) single genes do not necessarily reflect the historical record of the evolution of the organism; and (b) larger data sets (in terms of sequence length) increase phylogenetic accuracy both by increasing signal and dispersing noise.

5.2 Phylogeny Based on Nuclear rRNA Sequences

The small subunit ribosomal RNA sequence has been a favorite for the study of phylogenetics in the fungi. It is available from a broad range of species, which is due to the

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ease with which it can be amplified by PCR from tiny amounts of total DNA. However, there are distinct limitations to the use of this data. Although it often produces high bootstrap support for the resolution of certain nodes, there are many regions of fungal phylogenies, even at higher taxonomic levels, that remain unresolved with this data alone.

For example, Nishida and Sugiyama (1993), with the purpose of placing the genera *Taphrina* and *Saitoella* on the fungal tree, published a phylogenetic analysis of the Ascomycota based on rRNA sequences. Using distance methods without among-site variation, they concluded that the monophyletic group formed by *Saitoella complicata*, *Taphrina wiesneri*, and the fission yeast *S. pombe* branches at the base of the Ascomycota. However, when we re-analyzed the same data by the more reliable maximum likelihood method and by employing gamma distribution to account for among-site rate heterogeneity, a substantially different result is obtained (tree not shown). In our maximum likelihood tree, among other topological differences, the clade formed by *S. pombe*, *S. complicata*, and *T. wiesneri* is placed at the base of a group formed by the Ascomycota and the Basidiomycota (i.e., prior to the Basidiomycota–Ascomycota divergence; significantly supported with $P < 0.05$ in a weighted Shimodaira–Hasegawa test; Shimodaira (1999); see Table 2A).

Further, Philippe and Germot (2000) have shown that a distant outgroup tends to cause the attraction of longer branches towards the base of the tree. As only a single and distant species, *Oryza sativa*, was used as the outgroup of the analyses discussed previously, the unorthodox topology likely results from an inappropriate choice of taxa. This difficulty was addressed in a following publication by Nishida and Sugiyama (1994); however, bootstrap support remained marginal, and only a distance approach was used in this analysis. For this reason, we assembled a modified small subunit rRNA data set with an outgroup composed of several fungal species including three basidiomycetes, three zygomycetes, and a chytridiomycete. In addition, several of the taxa with longer branches were replaced by neighboring taxa with shorter branches, and the sequence alignment obtained was further refined manually based on the newly added data. The resulting topology that we obtained by maximum likelihood was similar to that of Nishida and Sugiyama in that the archiascomycetes (*S. pombe*, *Neolecta vitellina*, *T. wiesneri*, and *S. complicata*) were placed at the base of the Ascomycota (Figure 1). However, the bootstrap support for the monophyly of the archiascomycetes was extremely low (29%) and the support for the Ascomycota as a group was only 79%. Support values for many other branches of the tree were also very low. Likelihood tests of alternative evolutionary scenarios involving the placement of *S. pombe* indicate that most alternative topologies cannot be rejected, as confidence values are greater than 0.05 (Table 2B). Our results not only question the phylogenetic placement of *S. pombe*, but also the value of rRNA sequence data for fungal phylogenetic analyses at this evolutionary depth. The existence of a monophyletic archiascomycete lineage and the validity of the classification scheme currently used in GenBank that is based on the outcome of previously published rRNA phylogenies are therefore also questionable.

These and other results call for the use of more data than the 18S rRNA alone, for instance by combining it with large subunit rRNA data. Berbee et al. (2000) set out to determine how much additional sequence data would theoretically be required to resolve the major branching patterns. In line with our results presented here, they conclude that resolution of certain nodes would require much more sequence data than

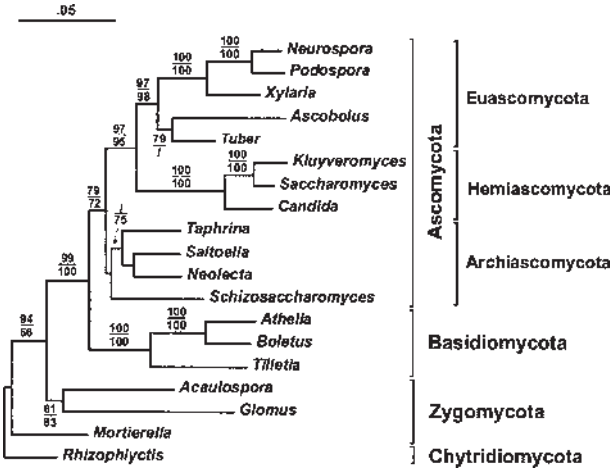

Figure 1 *Phylogenetic analysis based on small subunit rRNA sequences*. Only unambiguously aligned positions without gaps were considered for the analysis, a total of 1423 nucleotides. The tree topology was inferred using DNAML (Felsenstein, 2002) using the F84 model of nucleotide substitution; branch lengths were determined using BaseML, included in PAML package (Yang 1997), using the TN93 model (Tamura and Nei 1993). The alpha value was 0.25. Bootstrap support (%, upper number) was calculated from 100 replicates using SEQBOOT, provided with the PHYLIP package. Bootstrap support (%, lower number) was also calculated using DNADIST (Felsenstein 2002) and the F84 maximum likelihood model for nucleotide substitution, and Weighbor (Bruno et al. 2000). Maximum likelihood and distance bootstrap values are not indicated in cases where the bootstrap consensus topology differed from the maximum likelihood topology, or where values were less than 50%. Both distance and maximum likelihood trees are poorly supported, particularly in the group formed by the Archiascomycota. The ribosomal RNA sequences were retrieved from Genbank: *N. crassa* (X04971), *P. anserina* (X54864), *X. carpophila* (Z49785), *A. lineolatus* (L37533), *T. excavatum* (X98089), *K. lactis* (X51830), *S. cerevisiae* (J01353), *C. albicans* (X53497), *S. complicata* (D12530 D01174), *T. wiesneri* (D12531 D01175), *N. vitellina* (Z27393), *S. pombe* (X54866), *A. bombacina* (M55638), *B. satanas* (M94337), *T. caries* (U00972), *A. trapper* (Y17634), *G. caledonium* (Y17653), *M. polycephala* (X89436), *R. rosea* (AH009028).

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Table 2B Likelihood tests of alternative tree topologies based on small subunit rRNA (Figure 1)

Tree topology	Dli	NP	AU	wKH	wSH
Best tree (Figure 1)	−0.4	**0.435**	**0.822**	**0.640**	**0.981**
(a) yeasts/euascos monophyl, archis ancestral	0.4	**0.239**	**0.604**	**0.360**	**0.875**
(b) yeasts/euascos monophyl, *Sp* ancestral	0.5	**0.160**	**0.443**	**0.316**	**0.837**
(c) yeasts/euascos monophyl, archis/*Sp* monophyl, below basidios	7.5	**0.114**	**0.124**	**0.136**	**0.263**
(d) yeasts/*Sp* monophyl, archis ancestral	12.8	0.024	**0.114**	**0.056**	**0.210**
(e) euascos/archis monophyl, yeasts/*Sp* monophyl	14.7	0.016	**0.087**	0.047	**0.195**
(f) archis/*Sp* monophyl, on euasco branch, yeasts ancestral	14.7	0.003	0.046	0.025	**0.109**
(g) euascos/*Sp* monophyl, archis ancestral	14.8	0.001	0.011	0.027	**0.116**
(h) yeasts/*Sp* monophyl, archis ancestral	15.2	0.003	0.027	0.037	**0.197**
(i) archis/*Sp* monophyl, on yeast branch, euascos ancestral	15.9	0.002	0.019	0.020	**0.087**
(j) euascos/archis monophyl, *Sp* ancestral	16.3	0.002	0.032	0.026	**0.138**
(k) archis/euascos monophyl, yeasts ancestral	16.7	0.001	0.008	0.024	**0.152**
(l) archis/yeasts monophyl, *Sp* ancestral	16.9	0.000	0.014	0.019	**0.129**
(m) euascos/*Sp* monophyl, yeasts ancestral	17.3	0.000	0.007	0.018	**0.124**
(n) yeasts/archis monophyl, euascos/*Sp* monophyl	17.3	0.000	0.007	0.018	**0.124**
(o) yeasts/archis monophyl, euascos ancestral	17.6	0.001	0.031	0.015	**0.141**

Statistical tests to evaluate the phylogenetic placement of *S. pombe* (*Sp*). Tests were performed as outlined in Table 2A. The alternative scenarios include all possible arrangements of the euascomycetes (euascos), the hemiascomycetes (yeasts), the group formed by the archiascomycetes *T. wiesneri*, *N. vitellina*, and *S. complicata* (archis), and the fission yeast, a total of 15 possible topologies. In addition, a sixteenth topology, tree (c), in which *S. pombe* was placed with the archiascomycetes, and the basidiomycetes (basidios) were placed at the base of the group formed by the euascomycetes and the hemiascomycetes (i.e., the archiascomycetes were placed below the basidiomycetes) was tested. The wSH test does not warrant the rejection of any topology, as all p-values are greater than the confidence level (0.05; marked in bold). While the results of the other tests were better, the confidence set (i.e., all topologies that cannot be rejected) still contains either four (NP), five (wKH), or six (AU) topologies. Note that according to Shimodaira (2002), and our own experience, the AU test is most adequate for general tree testing. Tree (c), in which the basidiomycete and ascomycete lineages were not separated into distinct clades, was within the confidence set according to all four tests. Clearly, the small subunit rRNA data set used here cannot with certainty determine the phylogeny of the Ascomycota.

Note: "monophyl" indicates that the groups or species form a monophyletic group; "ancestral" indicates that a species or group is placed at the base of the ascomycetes. In some tree descriptions, the position of one of the four groups is not indicated. In these cases, the position of the fourth group can be deduced from the positions of the other three groups. For example, in tree (a), "yeasts" and "euascos" are monophyletic, and "archis" are at the base of the ascomycetes. "*Sp*" is placed above "archis," but not within the monophyletic group of "yeasts" and "euascos."

would be available even if the sequences of the small subunit and large subunit rRNAs were combined.

5.3 Phylogenies Based on Nuclear-Encoded Protein Sequences

Many of the problems of rRNA phylogenies might be solved with protein sequence data. Sequence conservation at the protein level is little biased by the nucleotide composition of genes. In addition, the large number of protein-coding genes present in nuclear genomes allows concatenation of amino acid sequences from a large number of genes, making these data suitable for resolution of the deepest divergences. However, there is currently little nuclear protein sequence data available in the fungi, and that which is available is predominantly concentrated in the Ascomycota. In fact, the distribution of available protein data among species is so biased that most published analyses are based on only single proteins.

The analysis of RNA polymerase II subunit encoded by the beta subunit of RNA polymerase II (RPB2) using distance methods produced a tree with high bootstrap support (Liu et al. 1999). The topology inferred was similar to that of Nishida and Sugiyama (1993) as well as small subunit rRNA-based trees published by others, but the bootstrap support of the RPB2 tree was considerably better. However, our re-analysis of the RPB2 data from the same species (excluding several species with long branches to reduce long branch attraction artifacts, and including additional species as an outgroup) produced a slightly different topology from that inferred by Liu et al, and many branches had only weak bootstrap support (Figure 2). In addition, likelihood tests of many different internal branches failed to reject many of the alternate topologies (Table 2C). The RPB2 protein data alone provide significantly better resolution than the nuclear rRNA data, but still leave much to be desired.

Lack of data is not the only problem in protein-based phylogenies. Certain proteins evolve at vastly unequal rates, producing branches of very different lengths. As mentioned before, uneven branch lengths tend to lead to long-branch attraction, even when using the maximum likelihood method for tree inference (Philippe and Germot 1998). In addition, it is sometimes difficult to determine which duplicated genes

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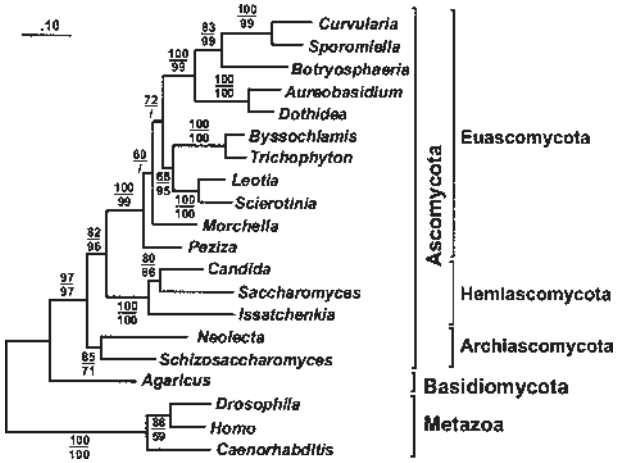

Figure 2 *Phylogenetic analysis based on the beta subunit of RNA polymerase II.* Only unambiguously aligned sequence positions without gaps were used for this analysis, producing a data set of 822 amino acid positions. The topology shown was inferred using ProML (Felsenstein, 2002) and the PMB model of protein evolution (Tillier, unpublished), and branch lengths were determined using CodeML, included in the PAML package (Yang 1997). The alpha value used was 0.5. Bootstrap support (%, upper number) was calculated from 100 replicates using SEQBOOT, provided with the PHYLIP package. A similar topology was obtained using ProtDist and Weighbor (lower numbers), except for the position of *Peziza quelepidotia* and *Morchella elata*, which were found to be monophyletic in the distance tree, branching at the base of the group formed by *B. nivea*, *T. rubrum*, *L. viscosa*, and *S. sclerotiorum*, with reasonably good bootstrap support (87%). Both distance and maximum likelihood based phylogenies contain several weakly supported branches. The protein sequences were retrieved from Genbank: *C. brachyspora* (AAF19075), *S. minima* (AAF19077), *B. rhodina* (AAF19074), *A. pullulans* (AAF19071), *D. insculpta* (AAF19072), *B. nivea* (AAF19066), *T. rubrum* (AF107795), *L. viscosa* (AAF19067), *S. sclerotiorum* (AAF19080), *M. elata* (AAF19082), *P. quelepidotia* (AAF19081), *C. albicans* (AAF19059), *S. cerevisiae* (AAA68096), *I. orientalis* (AAF19060), *N. vitellina* (AAF19058), *S. pombe* (Q02061), *A. bisporus* (AAF19057), *D. melanogaster*, (NP_476706), *H. sapiens* (NM_000929), *C. elegans* (NP_498047).

are truly homologous and can therefore be used for phylogenetic inferences (e.g., tubulins; Keeling and McFadden 1998). Baldauf et al. (2000) have published a kingdom-level phylogeny of eukaryotes using four concatenated nuclear proteins sequences: α-tubulin, β-tubulin, actin and elongation factor 1-alpha. Because tubulin data are difficult to interpret as they form a protein family, but also because of their high evolutionary rate variation in fungi, we assembled a new data set combining only the elongation factor 1-alpha and actin, which were available for a total of 14 fungal species from the Ascomycota, Basidiomycota, and

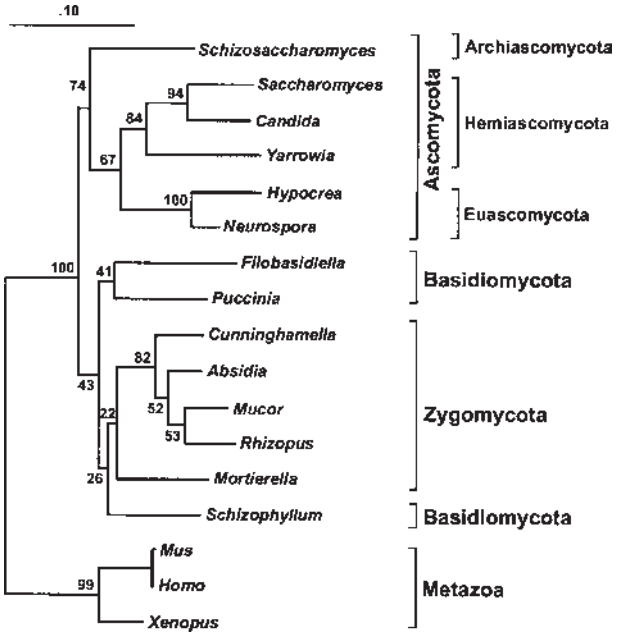

Figure 3 *Phylogenetic tree based on the concatenated nuclear proteins actin and elongation factor 1-alpha.* This maximum likelihood tree was inferred using ProML, using a total of 648 amino acid positions. The PMB substitution matrix was chosen as the model for protein evolution (Tillier, unpublished). To account for heterogeneity of amino acid substitution rates, a gamma distribution model was implemented, for which the regional rate categories option was selected. The optimal rates and probabilities for 6 categories were determined using PAML (Yang 1997). The maximum likelihood bootstrap values for 100 replicates are shown for all nodes. The scale bar indicates the average number of substitutions per amino acid. Multiple protein alignments were determined using CLUSTAL W (Thompson et al. 1994) which was executed from GDE (Smith et al. 1994) and further refined manually. Characters containing gaps were excluded for the inference of the tree. Sequences used (Genbank accession numbers of actin and elongation factor 1-alpha, respectively): *S. pombe* (NP_595618, Q10158); *S. cerevisiae* (ATBY, NP_009676); *C. albicans* (P14235, P16017); *Y. lipolytica* (Q9UVF3, O59949); *H. jecorina* (Q99023, P34825); *N. crassa* (P78711, Q01372); *F. neoformans* (P48465, O42671); *P. graminis* (P50138, P32186); *C. bertholletia* (CAC28239, AAG28993); *A. glauca* (CAC28231, P28295); *M. racemosus* (CAC28274, P14865); *R. stolonifer* (CAC28295, AAG29040); *M. polycephala* (CAC28272, AAG29011); *S. commune* (AAD38853, O42820); *M. musculus* (NP_033739, NP_031932); *H. sapiens* (AAA51579, NP_001949); *X. laevis* (AAC27796, P17508).

Zygomycota (the Chytridiomycota were not represented). The tree that we obtained using the maximum likelihood method is quite unorthodox, and contradicts the published results although with virtually no bootstrap support (Figure 3).

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Table 2C Likelihood tests of alternative tree topologies based on RPB2 (Figure 2)

Tree toplogy	Dli	NP	AU	wKH	wSH
Best tree (Figure 2)	−8.5	**0.746**	**0.951**	**0.884**	**0.997**
(a) yeasts/euascos monophyl, *Nv* ancestral	8.5	**0.072**	**0.143**	**0.111**	**0.388**
(b) yeasts/euascos monophyl, *Sp* ancestral	8.7	**0.065**	**0.171**	**0.105**	**0.378**
(c) *Sp*/*Nv* monophyl, on yeast branch, euascos ancestral	11.8	**0.099**	**0.180**	**0.116**	**0.386**
(d) *Sp*/*Nv* monophyl, on euasco branch, yeasts ancestral	15.8	0.009	0.029	0.037	**0.154**
(e) yeasts/*Nv* monophyl, euascos ancestral	28.8	0.004	0.027	0.024	**0.136**
(f) yeasts/*Nv* monophyl, *Sp* ancestral	29.0	0.005	0.025	0.015	**0.094**
(g) yeasts/*Sp* monophyl, euascos ancestral	29.9	0.002	0.021	0.019	**0.115**
(h) euascos/*Nv* monophyl, *Sp* ancestral	30.9	0.000	0.009	0.008	**0.056**
(i) yeasts/*Sp* monophyl, *Nv* ancestral	33.1	0.001	0.005	0.008	**0.060**
(j) euascos/*Sp* monophyl, *Nv* ancestral	34.4	0.000	0.000	0.005	0.041
(k) yeasts/*Sp*monophyl, euascos/*Nv* monophyl	35.0	0.000	0.007	0.006	**0.059**
(l) yeasts/*Nv* monophyl, euascos/*Sp* monophyl	35.3	0.000	0.005	0.006	**0.056**
(m) euascos/*Nv* monophyl, yeasts ancestral	35.7	0.000	0.005	0.004	0.034
(n) euascos/*Sp* monophyl, yeasts ancestral	36.9	0.000	0.020	0.003	0.033

Statistical tests to evaluate the phylogenetic placement of the species *N. vitellina* and *S. pombe* among the ascomycetes. Tests were performed as outlined in Table 2A. All possible topological rearrangements of the groups formed by the hemiascomycetes (yeasts) and euascomycetes (euascos), and the species *N. vitellina* and *S. pombe* were compared. Although the wSH test produced p-values greater than the confidence level of 0.05 for most of the alternative topologies, and therefore does not support the rejection of these topologies, the other statistical test results are less severe. NP, AU and wKH tests all produced a confidence set of only four topologies. However, we cannot conclude whether the archiascomycetes (*S. pombe* and *N. vitellina*) are, in fact, the earliest diverging group of the ascomycetes, or whether they are a sister clade to the hemiascomycetes, or whether they actually form a natural (monophyletic) group.

Note: "monophyl" indicates that the groups or species form a monophyletic group; "ancestral" indicates that a species or group is placed at the base of the ascomycetes. In some tree descriptions, the position of one of the four groups is not indicated. In these cases, the position of the fourth group can be deduced from the positions of the other three groups. For example, in tree (a), "yeasts" and "euascos" are monophyletic, and "*Nv*" is at the base of the ascomycetes. "*Sp*" is placed above "*Nv*," but not within the monophyletic group of "yeasts" and "euascos."

Table 2D Likelihood tests of alternate topologies based on mitochondrial proteins (Figure 4)

Tree topology	Dli	NP	AU	wKH	wSH
Best tree (Figure 4; yeasts/*Sp* monophyletic)	−17.6	**0.952**	**0.959**	**0.951**	**1.000**
(a) yeasts/euascos monophyl, basidios below *Sp*	17.6	0.045	**0.067**	0.049	**0.192**
(b) *Sp*/euascos monophyl, basidios below yeasts	25.9	0.000	0.000	0.002	0.010
(c) yeasts/euascos monophyl, *Sp* below basidios	39.0	0.003	0.005	0.008	0.041
(d) yeasts/euascos monophyl, *Sp*/basidios monophyl	42.0	0.000	0.010	0.005	0.019
(e) yeasts/*Sp* monophyl, euascos below basidios	64.7	0.000	0.000	0.000	0.000
(f) yeasts/*Sp* monophyl, euascos/basidios monophyl	66.1	0.000	0.000	0.000	0.000
(g) yeasts/basidios monophyl, *Sp*/euascos monophyl	98.9	0.000	0.002	0.000	0.000
(h) yeasts/basidios monophyl, euascos below *Sp*	99.9	0.000	0.001	0.000	0.000
(i) yeasts/basidios monophyl, *Sp* below euascos	100.6	0.000	0.001	0.000	0.000
(j) euascos/basidios monophyl, *Sp* below yeasts	100.9	0.000	0.000	0.000	0.000
(k) *Sp*/basidios monophyl, euascos below yeasts	100.9	0.000	0.002	0.000	0.000
(l) euascos/basidios monophyl, yeasts below *Sp*	102.5	0.000	0.000	0.000	0.000
(m) *Sp*/euascos monophyl, yeasts below basidios	104.0	0.000	0.000	0.000	0.000
(n) *Sp*/basidios monophyl, yeasts below euascos	107.9	0.000	0.000	0.000	0.000

Statistical tests to evaluate phylogenetic placement of *S. pombe* (Sp) among the *Fungi*. Tests were performed as outlined in Table 2A. These tests evaluate all possible arrangements of the hemiascomycetes (yeasts), euascomycetes (euascos), basidiomycetes (basidios) and *S. pombe (Sp)*. While both NP and wKH tests rejected all alternative topologies, the AU and wSH tests produced p-values higher than the confidence level (0.05) for tree (a), in which *S. pombe* is placed at the base of the Ascomycota (as in the small subunit rRNA and RNA polymerase trees). However, we do obtain significant support for only the best tree, and with all test methods, when adding further archiascomycete species to our data set (unpublished results).

Note: "monophyl" indicates that the groups or species form a monophyletic group.

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The basidiomycetes are paraphyletic, and basidiomycetes plus zygomycetes form a monophyletic group to the exclusion of ascomycetes. We also obtained similarly unorthodox results when using maximum likelihood without gamma distribution, quartet puzzling (Strimmer and von Haeseler 1996), and distance methods (data not shown). Bootstrap analysis using maximum likelihood showed little support for the majority of the branches, even at some higher taxonomic levels. Clearly, significantly more protein data than is available from these two genes is required for a satisfactory phylogenetic analysis of fungi.

5.4 Phylogenies Based on Mitochondrial Protein-Coding Genes

Because of their small size and usually high gene density, complete mtDNA data can be produced at reasonable cost. In fungal phylogenies, mitochondrial protein data has produced unprecedented resolution for many branches. Figure 4 shows the results of our analysis of concatenated mitochondrial proteins Cox1,2,3, Cob, Nad1,2,3,4,4L,5 and Atp6,9 by the maximum likelihood method, using the PMB substitution matrix (Tillier, unpublished), and a gamma distribution model to account for heterogeneity of amino acid substitution rates across sites. The resulting tree has far greater bootstrap support than was produced by any of the other data sets shown so far. In this tree topology, the position of *S. pombe* at the base of the budding yeasts (Hemiascomycota) is inconsistent with both rRNA- and RPB2-based phylogenies, although consistent with a phylogeny based on multiple nuclear proteins (Baldauf et al. 2000). Although the bootstrap support for this position of *S. pombe* is high in our analysis (99 and 98%, for ML and distances analyses respectively), we were unable to reject its placement at the base of the ascomycetes with a confidence value of less than 0.05 (based on the AU and wSH tests; see Table 2D). Although both *S. pombe* and budding yeasts have long branches in our mitochondrial analyses that tend to regroup them artificially, we are fairly convinced of the position of *S. pombe* at the base of the yeasts, as we have used maximum likelihood inferences for our analyses. In addition, the inclusion of sequences from other archiascomycete species further stabilizes this tree topology (unpublished results).

As previously noted (Forget et al. 2002) there is now only weak support for the position of *A. macrogynus* at the base of the Zygomycota–Basidiomycota–Ascomycota group when using distance methods on our mitochondrial data set. This casts doubt on the paraphyly of the Chytridiomycota, as proposed by us several years ago (Paquin and Lang 1996; Paquin et al. 1997; however, this suggestion was based on limited data and without applying a gamma distribution model. With maximum likelihood, a more appropriate evolutionary model, and a larger data set, we now observe monophyly of the Chytridiomycota, although only with

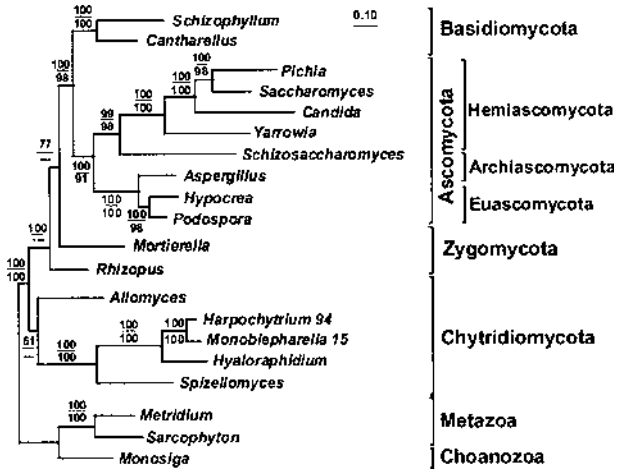

Figure 4 *Phylogenetic analysis based on concatenated mitochondrial proteins*. The phylogenetic tree was constructed from unambiguously aligned portions of the concatenated protein sequences of Cox1, Cox2, Cox3, Cob, Atp6, Atp9, Nad1, Nad2, Nad3, Nad4L, Nad4, Nad5, a total of 2829 amino acid positions. The tree was constructed following the same procedures as described in Figure 2, except that an alpha factor of 1.23 was chosen, and distance tables were calculated using maximum likelihood (PUZZLE). Because distance methods are sensitive to lack of sequence information, while ML methods are not, we chose to use the full data set of 2829 amino acid positions only for the inference with ProML. For the distance tree, we eliminated all Nad protein sequences from the alignment (the corresponding genes are not present in *S. cerevisiae* and *S. pombe*), leaving a total of only 1427 amino acid positions. The tree topologies were similar with both methods, except: (a) in the distance tree, the Zygomycota were found to be monophyletic, with 97% bootstrap support, while they are paraphyletic in the ML analysis, and (b) in the distance method-based tree the Chytridiomycota are paraphyletic, with *A. macrogynus* branching at the base of the Zygomycota–Ascomycota–Basidiomycota group, though this result is not supported (57%). In the ML analysis, the Chytridiomycota are monophyletic, although also with marginal support (61%). More importantly, trees inferred by both methods place *S. pombe* at the base of the Hemiascomycota, with high bootstrap support (100% in maximum likelihood, 98% in distance), a result that is incompatible with trees based on the small subunit rRNA (Figure 1) and RPB2 proteins (Figure 2). Sequences were obtained from Genbank: *S. commune* (AF402141), *P. canadensis* (D31785), *S. cerevisiae* (AJ011856), *C. albicans* (AF285261), *Y. lipolytica* (AJ307410), *S. pombe* (X54421), *H. jecorina* (AF447590), *A. nidulans* (ODAS1, CAA33481, AAA99207, AAA31737, CAA25707, AAA31736, CAA23994, X15442, P15956, CAA23995, CAA33116X00790, X15441, X06960, J01387, X01507), *P. anserina* (X55026), *A. macrogynus* (U41288), *H. curvatum* (AF402142), *S. punctatus* (AF402142), *M. senile* (AF000023), *S. glaucum* (AF064823, AF063191). Protein sequences of *C. cibarius*, *M. brevicollis, Harpochytrium 94*, *Monoblepharella 15*, *R. stolonifer*, and *M. verticillata*can be downloaded from http://megasun.bch.umontreal.ca/People/lang/FMGP/proteins/.

marginal bootstrap support (Figure 4) and without significant distinction by the AU test.

6 CONCLUSIONS

It is difficult to appraise our current knowledge of the evolutionary relationships among the Fungi without pointing out the high degree of inconsistency among published phylogenetic analyses and the lack of statistical support for most of them. Fungal phylogenetics continues to be in a state of chaos, and it will take substantial genome sequencing efforts to address this question with sufficient data. The few virtually undisputed results are summarized below.

Phylogenetic analyses with nuclear rRNA sequences, 70 kDa heat-shock protein (HSP70), elongation factor 1α, actin, α-tubulin and (β-tubulin, as well as mitochondrial protein sequences, provide a clear-cut consensus and high support for the sister relationship of Metazoa and Fungi (as listed in Table 1). Accordingly, the monophyly of these two kingdoms justifies their grouping into the superkingdom Opisthokonta (Cavalier-Smith and Chao 1995). The last common ancestor of Metazoa and Fungi is thought to have been a unicellular flagellated protist similar to the extant choanoflagellates (e.g., *Monosiga* spp.), or the Mesomycetozoa that include species such as *Dermocystidium* spp. or *Ichthyophonus* spp. The divergence of fungi and animals is estimated to have occurred approximately 1 BYA ago (Doolittle et al. 1996).

Currently only mitochondrial data sets provide sufficient support in the resolution of the branching order of the main fungal phyla in global phylogenetic analyses by using multiple protein sequences (e.g., Forget et al. 2002; see also Figure 4). In contrast, a phylogeny that was published recently based on four concatenated nuclear proteins is only weakly supported for most fungal branches (Baldauf et al. 2000). Contrary to common views, the latter phylogeny postulates that Archiascomycota and Hemiascomycota are sister groups, and that Basidiomycota are a sister lineage to the Zygomycota.

Ideally, to resolve the fungal tree at some depth, phylogenetic analyses should include multiple protein sequences from three or more distant species for every major fungal clade, which would sum up to a total of at least 30 species. The available mitochondrial data closely approaches this distribution and number of species (http://megasun.bch.umontreal.ca/People/lang/FMGP/progress.html), except that it is limited by the number of available genes that occur in mitochondria; moreover, that mtDNA is absent in the known Neocallimasticales (Chytridiomycota). It remains to be seen whether this data set has enough phylogenetic signal to resolve, beyond any reasonable doubt, divergences up to 1 BYA (for example, the question of whether the Chytridiomycota are monophyletic or paraphyletic; Paquin and Lang, (1996); Paquin et al. (1997). For the derived mtDNAs of *S. cerevisiae* and *S. pombe* the situation is further complicated by the fact that they do not carry any of the otherwise omnipresent and phylogenetically useful *nad* genes coding for subunits of the NADH dehydrogenase complex. This deficiency significantly reduces the phylogenetic signal in analyses that include these species.

There are other solutions to obtaining sufficient data for phylogenetic inferences, such as sequencing several dozen fungal nuclear genomes, or alternatively, sequencing several thousand cDNAs for each species. Several genomics initiatives are currently underway that will ultimately elucidate fungal evolution from a genomics point of view. For instance, sequencing of chromosome 7 (4.2 Mb) of the fungus *Magnaporthe grisea*, a pathogen of rice responsible for the annual loss of 157 million tons of rice worldwide, is currently in progress. Sequencing of ESTs (cDNAs) from the fungus *Fusarium graminearum* is also underway, and various biotechnology companies are in the process of determining complete genome sequences for the fungi *Ustilago maydis*, *Cochliobolus heterostrophus*, and *Botrytis cinerea* (Pennisi (2001)). Finally, the "Fungal Genome Initiative" proposed the sequencing of 15 fungal genomes relevant to the field of medecine, agriculture, industry, comparative genomics, and evolution. This project was recently given high priority status by the GRASPP at the National Human Genome Research Institute (http://www.genome.gov). Out of the 15 proposed fungi, the GRASPP suggested sequencing only of 4 ascomycete and 3 basidiomycete species (*Cryptococcus neoformans* Serotype A, *P. carinii*, *M. grisea*, *A. nidulans*, *F. graminearum*, *C. cinereus* and *U. maydis*). The GRASPP has encouraged additional thought regarding the choice of other species to be sequenced. Sequencing should start at the institute-supported centers as capacity becomes available.

Unfortunately, the selection of species continues to be highly biased towards economically relevant fungi, and towards ascomycetes and basidiomyctes. In the long run, genome sequencing in lower fungi should be given highest priority, not only with the goal of solving phylogenetic questions, but also to realize the full potential of fungal genomics and the development of new model systems.

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Glossary of Phylogenetic Terms

Synapomorphy, a derived character or character state, shared among a group of species to the exclusion of others.

Bootstrap analysis, technique to evaluate the robustness of a tree. The original data set is randomly sampled to generate a new data set of identical size in which some characters are resampled several times whereas others are absent. The new data set is then used for tree-building. After repeating the process many times, a majority-rule consensus tree is calculated, in which the support for an internal branch is expressed as the number of times it was recovered.

Character, any heritable attribute of organisms that varies among species, and that can be used for phylogenetic inference.

Homology, Similarity due to common evolutionary origin, i.e., derivation from the same ancestral character.

Jackknife analysis, a resampling technique related to boostrap analysis that involves the deletion of characters from the original data set to produce a data set that is a fraction the size of the original (e.g., 50%).

Likelihood test, maximum likelihood-based test assessing the significance of differences among competing phylogenetic hypotheses.

Maximum likelihood, optimizes the statistical likelihood for a specified evolutionary model of character changes.

Monophyletic group, a monophyletic group (clade) has a unique origin in a single ancestral species, and includes the ancestor and all of its descendants.

Outgroup, taxon (taxa) used to root a phylogenetic tree, and that is (are) considered to lie outside of the group of interest.

Paralogy, Paralogous genes result from duplication, and will reconstruct gene phylogeny rather than species phylogeny.

Paraphyletic group, a group consisting of a common ancestor and some but not all of its descendants.

Phylogenetics, Systematics that deals with evolutionary relationships among living organisms and their ancestors.

Parsimony analysis, inference method that minimizes the number of character transformations (e.g., nucleotide changes).

Polyphyletic group, a group of organisms descended from more than one common ancestor.

Root, see outgroup.

Phylogenetic tree (phylogeny), line graph representing the evolutionary history of a set of taxa, connecting contemporary taxa *via* internal branches to hypothetical ancestors.

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Genome Sequence Patterns and Gene Regulation: A Bioinformatics Perspective

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1 INTRODUCTION

Every cell in an organism contains the same genetic code, with the functional role of cell being controlled by differential gene expression. Protein coding regions in a eukaryotic DNA only account for 2% of the genome. However, noncoding nuclear DNA has been shown to be important for regulation of gene expression with the mechanisms of gene regulation being coordinated by the presence of biologically significant DNA sequence patterns observed within the noncoding in the neighboarhood of genes (Kliensmith and Kish 1995).

Several sequences of regulatory importance, including the introns, promoters, enhancers, matrix/scaffold attachment regions (S/MARs), repeats, etc., have been observed in the noncoding DNA (Hughes et al. 2000). Some of these regions represent patterns that serve as functional control points for cell specific or differential gene expression, while others such as the repetitive DNA serve as a biological clock (Hartwell and Kasten 1994). Rare occurrence or absence of dinucleotides TA and GC, and tetranucleotide CTAG, and the GNN periodicity have been observed within the regions coding for proteins (Kadonaga 1998). Other example patterns include the A + T or G + C rich regions, telomeric repeats of sequence AGGGTT. The DNA is therefore comprised of a mosaic of sequence level motifs that come together in a synergistic manner to coordinate and regulate in synthesis of proteins (Fickett and Wasserman 2000). Successful transcription or the synthesis of RNA from the DNA template proceeds in the following phases:

(a) *Potentiation or the structural DNA conformation*. This step is a prerequisite for expression where the potentiated genes are those that are located on the 10 nm fiber. Higher-level (or functional) patterns described as matrix/scaffold attachment regions (S/MARs) are responsible for maintaining the regions of the genome in a *potentiated* conformation.

(b) *Initiation or binding of RNA polymerase to double-stranded DNA*. This step involves results of binding of RNA polymerase to the promoter region of the single-stranded DNA. Consensus DNA sequence patterns observed on the promoter regions has been documented for both prokaryotes and eukaryotes.

(c) *Elongation* or the covalent addition of nucleotides to the 3′-end of the growing polynucleotide chain. Patterns for basal and inducible transcription factors are responsible for gene regulation and differential expression.

(d) *Termination* or the recognition of the transcription termination sequence and the release of RNA polymerase. Polyadhenylation signals and stop codons are two examples of DNA sequence patterns that facilitate in this process.

Our focus in this chapter is to investigate patterns from a quantitative perspective and discuss the statistical underpinnings of the pattern detection tools in bioinformatics. Furthermore, the ideas presented would be applied for the detection of regions with high transcription potential as well as those with high potential for nuclear matrix attachment.

2 GENOMIC SEQUENCE PATTERNS

Numerous examples indicate that patterns in the eukaryotic DNA play an important role in the organism's viability. The mathematical models designed for modeling the biological

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sequence and those for the detection of patterns are therefore of fundamental importance in bioinformatics. Patterns are often organized hierarchically. For example, patterns at the lowest levels are sequence levels motifs representing the splice sites, binding sites, and domains. These are subsequently utilized for the definition of patterns at a higher level of abstraction such as introns, exons, repetitive DNA, and locus control regions (LCRs).

2.1 Transcription Factors

The role of transcription factors as sequence level patterns significant for gene transcription has been well documented (Wingender 1994). The transcription factors are categorized into three major groups, namely, basal, upstream, and inducible. First, the basal transcription factors are needed by all genes and are essential for the very process of transcription. The TATA-box and CAAT-box are examples of basal factors. Secondly, the upstream transcription factors affect the efficiency of transcription. For example, the nuclei of the cells that contain the upstream factors are able to synthesize the gene in larger numbers. Thirdly, the inducible transcription factors regulate the expression of a gene in response to various stimuli as well as during growth and development.

The basal transcription apparatus required for the transcription of the protein-coding genes (i.e., those transcribed by RNA Polymerase II), exhibits similarity to the prokaryotic as well as eukaryotic genes. In general, the prokaryote promoters are turned on, and control is exerted in a negative manner, whereas, a typical eukaryotic promoter will carry out basal level transcription and require positive activators for efficient transcription. These upstream transcription factor bindings generally enhance the levels of transcription and assist in transcription initiation by recruiting coactivators to the basal apparatus (Wingender et al. 1997).

The group of promoters, known as upstream promoters, is diverse. This makes the combinations of their co-occurrences is quite large and enables the cellular machinery to exhibit a sophisticated control on transcription. Transcription rates increase in the presence of these factors. In addition, a set of distant promoters, called the enhancers, alter the chromatin structure and influence gene expression. In addition to containing binding sites for constitutive transcription factors like TFIID, SP1, etc., the eukaryotic promoters contain sites for inducible transcription factors. These factors are turned on in response to the environmental stimuli and provide the final control point in regulating gene expression. Such factors will activate transcription of genes in response to growth factors, nutrient levels, heat shock, or other types of signals.

Transcription factors have also been classified based on the structural features of their DNA-binding domains. Such a categorization into superfamilies comprises of examples such as b/ZIP, bHLH, homeodomain proteins, ETS, REL, zinc finger nuclear proteins 2, the HMG-family the Winged Helix, serum response factors, and CTF/NF-1. Each superfamily is further subdivided into several families, and the proteins belonging to a specific family recognize cis-elements with distinct structure. For example, the families AP-1, CREB/ATF, C/EBP belong to the b/ZIP superfamily. Similarly, the EGR (early growth response gene) and Sp-1 (simian virus protein-1) families are members of C2H2 zinc finger superfamily. Such a diversity of transcription factors makes achieves a cell-specific differential gene regulation (Aach et al. 2000).

2.2 Matrix Attachment Regions

The structural properties of the DNA help in maintaining the chromatin in a potentiated conformation for a gene to be transcribed. The end-points of potentiated genes are known as matrix attachment regions or MARs (Nikolaev et al. 1996). The actual transcription is performed by transcription factors (Phi-Van and Strätling 1988).

DNA sequences are often modeled as probabilistic phenomena, with the patterns of interest being defined as samples drawn from the underlying random process. For example, the underlying DNA sequence is modeled as a Markov chain of random variables taking on the values $\{A, C, T, \text{and } G\}$. Given this underlying assumption, one may next model a splice site as a function P that assigns a sample sequence, S, of 8–10 nucleotides, a value equivalent to their probability of being a splice site (Mitchell and Tjian 1989).

The S/MAR are 100–1000 bp long sequences that anchor the chromatin loops to the nuclear matrix. The MARs often serve as the origins of replication (ORI). Often they are concentrated in the area of transcription factor binding sites. Out of a total of approximately 100,000 matrix attachment sites believed to exist in the mammalian nucleus, approximately 30,000–40,000 serve as ORIs. Other MARs have been observed to flank the ends of genic domains encompassing various transcriptional units. The MARs are thought to bring together the transcriptionally active regions of chromatin such that transcription is initiated in the region of the chromosome that coincides with the surface of nuclear matrix (Bode et al. 1995; Bode et al. 1996).

There are two potentiated states of open euchromatin, i.e., constitutive and tissue/cell-specific or facultative. The constitutive classes, such as those of the housekeeping genic domains, are always maintained in transcriptionally favorable open chromatin conformation. In contrast, the tissue/cell-specific or facultative classes impose the open conformation onto a segment of the genome in a tissue/cell-specific manner. The two potentiative states, ready/open or off/closed, are correlated with the presence or absence of multiple factors interacting with element(s) that are far distal of their respective gene-specific promoters and enhancers at regions of locus control.

Three known classes of elements acting as regions of locus control have been identified, suggesting that potentiation may occur with multiple and/or redundant means. The classes of elements are MARs/SARs or the matrix/scaffold attachment

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regions; SCSs or specialized chromatin structures; and LCRs or the locus control regions. These elements provide a dominant chromatin opening function that is an absolute requisite for transcription of this segment of the genome. In this manner phenotype is ultimately defined by gene potentiation.

3 STATISTICAL MODELS FOR PATTERN DETECTION

Pattern detection algorithm must not only detect substring from a DNA sequence that match the prespecified pattern, but also help the biologists in estimating the significance of the detected patterns. The patterns that score highly on the "significance" criteria may be further investigated using the appropriate wet-bench approaches. The underlying model defining the DNA sequences is ultimately the determinant of the significance of the patterns detected. Thus, the modeling of the sequences and that of patterns are two complementary objectives, each playing an important role in the bioinformatics discovery process. Sequence models provide a basis for establishing significance of patterns observed, while the pattern models help us look for specific motifs that are of functional significance. Both these issues are discussed below.

3.1 Sequence Models

The two main sequence models are the independent identically distributed (IID) and Markov Chain models. Sequence models represent the background stochastic processes in a manner that enables one to analytically justify the significance of an observation. To provide an analogy, the determination of the sequence model is similar to determining the probability of obtaining a *head* (H) while tossing a coin. For a fair coin, this probability would be $\frac{1}{2}$. We may estimate this probability by studying the strings of the heads and tail sequences that a given coin has produced in the past. Similarly, given the DNA sequence(s), we may induce the underlying model that represents the density function produced the sequence considering that each base pair generated by the model is independent of the previous base (IID model) or depends on the previously produced base (Markov chain). We examine these modeling strategies in more detail below.

3.1.1 Independent Identically Distributed (IID) Model

The simplest of all the sequence models is the IID model. In this model, each of the four nucleotides is considered to occur independent of each other. Furthermore, the probability of occurrence of a given nucleotide at a given location is identical to the probability of its occurrence at another location. Thus, for example, assume that the sequence is defined on the DNA alphabet $\Sigma = \{A, C, T, G\}$. In this case, defining the individual probability values (p_A, p_C, p_T, and p_G) specifies the complete model for the sequence. The values may in turn be computed simply by considering the prevalence of each of base in the given sequence. In statistical terminology, the maximally likely (ML) estimator for probability of occurrence of a given base is X is simply n_X/L where n_X is the frequency of occurrence of the base X in a sequence of length L. In general, the probability of each base α, may be estimated as:

$$p_\alpha = \frac{n_\alpha}{|L|} \tag{1}$$

This simply counts the relative frequency of nucleotide in a sequence of length L. This estimator has the advantage of simplicity, and usually works well when L is large. Given the Model $\mathrm{M_{IID}}$ has been induced from the sequence data, the probability of an occurrence of a pattern x may be computed using the following:

$$P(x|\mathrm{M_{IID}}) = \prod_{i=1,\ldots,n(x)} P(x_i) \tag{2}$$

where $P(x_i)$ is the probability of nucleotide x_i at position i along the pattern. The model assumes that the parameters (probability of each of the four nucleotides) are independent of the position along the pattern. Other probability estimation methods have been proposed in Staden (1988a,b).

3.1.2 Markov Chain Model

In a Markov chain model, the base observed at position x along the sequence is dependent on the base at position $(x-1)$. The number of previous bases that influence the value of the base at a given location along the sequence is known as the degree of the Markov process. The first-degree Markov chain model has $|\Sigma| + |\Sigma|^2$ parameters, corresponding to the individual nucleotide frequencies as well as dinucleotide frequencies. This sequence model M is defined on the sample space Σ^* and assigns to every sequence x of length $n(x)$ on Σ^* a probability:

$$P(x|M) = P_1(x_1)\prod_{i=2,\ldots,n(x)} P_2(x_i|x_{i-1}) \tag{3}$$

where P_1 is a probability function on Σ that models the distribution of α is at the first position in the sequence and, P_2 is the conditional probability function on $\Sigma \times \Sigma$ that models the distribution of β's at position $i > 1$ on the alphabet symbol α at position $i-1$.

3.1.3 Higher Order Markov Models

Higher order Markov chains have been described. For example, the nth order Markov process has a memory of n, and thus the occurrence of a nucleotide depends on the previous n nucleotides. The probability of observing a sequence x is defined in a manner similar to the first order Markov chains.

$$P(x|M) = P_1(x_1)\prod_{i=2,\ldots,n(x)} P_2(x_i|x_{i-1},\ldots,x_{i-n}) \tag{4}$$

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The nth order Markov chain over some alphabet A is equivalent to a first order Markov chain over the alphabet A^n of n-tuples. This follows from the above discussion that this probability is given by Eq. (5).

$$P(x_k|x_{k-1},\ldots,x_{k-n}) = P(x_k,x_{k-1},\ldots,x_{k-n+1}|x_{k-1},\ldots,x_{k-n}) \tag{5}$$

That is, the probability of x_k given the n-tuple ending in x_{k-1} is equal to the probability of the n-tuple ending in x_k given the n-tuple ending in x_{k-1}.

3.2 Pattern Models

Having described some methodologies for modeling sequences, we now turn our attention toward describing the statistical modeling procedures for DNA patterns. There are a growing number of well-established patterns that we may wish to model and search for in DNA sequences. Often these patterns of functional significance are brought forth after an alignment of sequences belonging to a particular family. Such a multiple sequence alignment is often interspersed with gaps of varying sizes. However, there are sections in the final alignment that are free of gaps in all of the sequences. This fixed size ungapped aligned regions represent the types of patterns to model to identify in an anonymous segment of DNA. The statistical technique based on hidden Markov models (HMM) may be employed for developing such a closed form representation of a set of patterns is described below.

3.2.1 Regular Expressions

Regular expressions are utilized in bioinformatics for representation of patterns in a condensed format. One of the most useful features in Perl programming language is its powerful string manipulation features, including its ability to manipulate regular expressions—the natural representational style for biological patterns. The Perl programming language is popular with biologists because of its practical applications to the DNA and protein sequences. For example, a compact regular expression based representation of the TATA-box could be devised. Consider for example that the sequence level motifs for TATA-box are TATAAT or TATTAT or TATAA. One could conveniently represent and look for these variations using a single regular expression: TAT(AAT|TAT|AA).

3.2.2 Profiles

Profiles were the earliest representation methodology for capturing variability in the sequence data. Profiles or the{NBsp] profile matrix is often computed from the multiple sequence alignment of the patterns that we aim to represent. Multiple sequence alignments of the various variable forms of the{NBsp] pattern are used to find a profile matrix representing the{NBsp] statistical estimate of the probability of finding a base at a given location (Gribskov et al. 1987; Gribskov et al. 1990). It is possible to perform a comparison of a profile matrix against a sequence or series of sequences. The profile matrix specifies a different score for each letter (column) in each position in the alignment (row). This differs from scoring matrices, which assign a single score for a two-letter match independently of the match's position in the alignment. Pattern search methologies are devised to detect the pattern scores the highest using the weight assigned to each column of the pattern.

3.2.3 Hidden Markov Models

There are several extensions to the classical Markov chains, and the HMMs are one such extension. The rationale for building a hidden Markov model comes from the observation that as we search a sequence, our observations could arise from a model characterizing a pattern, or from a model that characterizes the background (Rabiner 1989). Hidden Markov DNA sequence models are developed to characterize a model as an *island* within the *sea* of nonisland DNA. In addition, the Markov chain characterizing both needs to be present within the same model, with the ability to switch from one chain to the other. In this manner, a HMM utilizes a set of hidden states with an emission of the symbols associated with each state.

From a symbol generation perspective, the state sequence executed by the model is not observed. Thus, the state sequence must be estimated from the observed symbols generated by the model. From a mathematical perspective, the HMM is characterized by the following parameters-Σ or an alphabet of symbols, Q or set of states that emit symbols from the alphabet Σ, $A = (a_{kl})$, which is a matrix of state transition probabilities, and $E = (e_k(b))$, a matrix of emission probabilities.

Although a general topology of a fully connected HMM allows the state transitions from any state to any other, this structure is almost never used. This is primarily due to the inadequacy of the available data for training a model with the large number of parameters needed for a fully connected HMM developed for any practical problem.

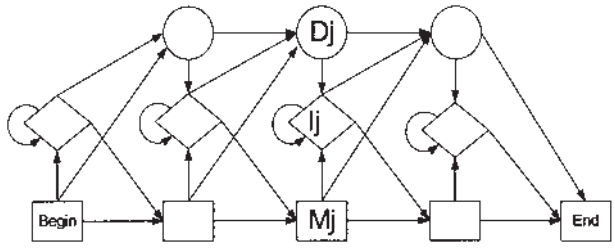

Figure 1 A Profile HMM utilizes the insert (diamond) and delete (circle) states. The delete states are silent and not associated with the emissions of any symbols. Profile HMM parameters are induced from multiple sequence alignments.

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Often, the over-generalized model produces suboptimal results due to the lack of training data. Consequently, more restrictive HMMs that rely on the problem characteristics to suitably reduce the model complexity and the number of model parameters that are needed are utilized. One such model is defined to be the profile-HMM, which is induced by a multiple sequence alignment (Krogh et al. 1994). The structure of a profile HMM is shown in Figure 1.

The parameters of a profile HMM are estimated using the sample alignments of the sequences used for training. The transitions and the emissions of the symbols in the alignment is used to derive the ML estimator of the HMM parameters. These values are assigned as shown in Eq. (6). The actual transition and emission frequencies A_{kl} and $E_k(a)$ respectively, are used to define the transition and emission probabilities, a_{kl} and $e_k(a)$. Furthermore, pseudo-counts are added to the observed frequencies to avoid zero probabilities. Simplest pseudo-count method is Laplace's rule, which requires adding a one to each frequency.

$$a_{kl} = \frac{A_{kl}}{\sum_X A_{kX}} \quad \text{and} \quad e_k(a) = \frac{E_k(a)}{\sum_Y E_k(Y)} \tag{6}$$

4 YEAST TRANSCRIPTION FACTORS

The 284 yeast transcription factor sequences in TFD were extracted and studied for their occurrence in Chromosome I. The results of the frequency profile are shown in Figure 2. This plot provides the relative occurrence of yeast transcription factors in Chromosome I on a logarithmic scale. As was expected, the frequency of shorter patterns increases linearly on the logarithmic scale. This is expected as probability of random occurrences of the patterns varies exponentially with pattern length.

We can further device a strategy for finding regions of this chromosome that have a high potential for participating in transcription regulation. A sliding window algorithm may be used for this purpose. The count of TFs found a window is used to assess the likelihood that the region participates in gene regulation. Since the probability of occurrence of a sequence pattern defined for the TF is inversely proportional to the length of the motif, we have further associated a weight with each TF pattern. This weight is proportional to the probability that the pattern occurs at random assuming an IID model for the background sequence. The regions for potential transcriptional activity are shown as the peaks in Figure 5.

5 MAR DETECTION

The human genome project (HGP) relies on the availability of databases and tools that enable easy access, analysis and comparison of genome information. As the sequencing phase of HGP nears the completion, the analysis paradigm is shifting towards developing computational tools and

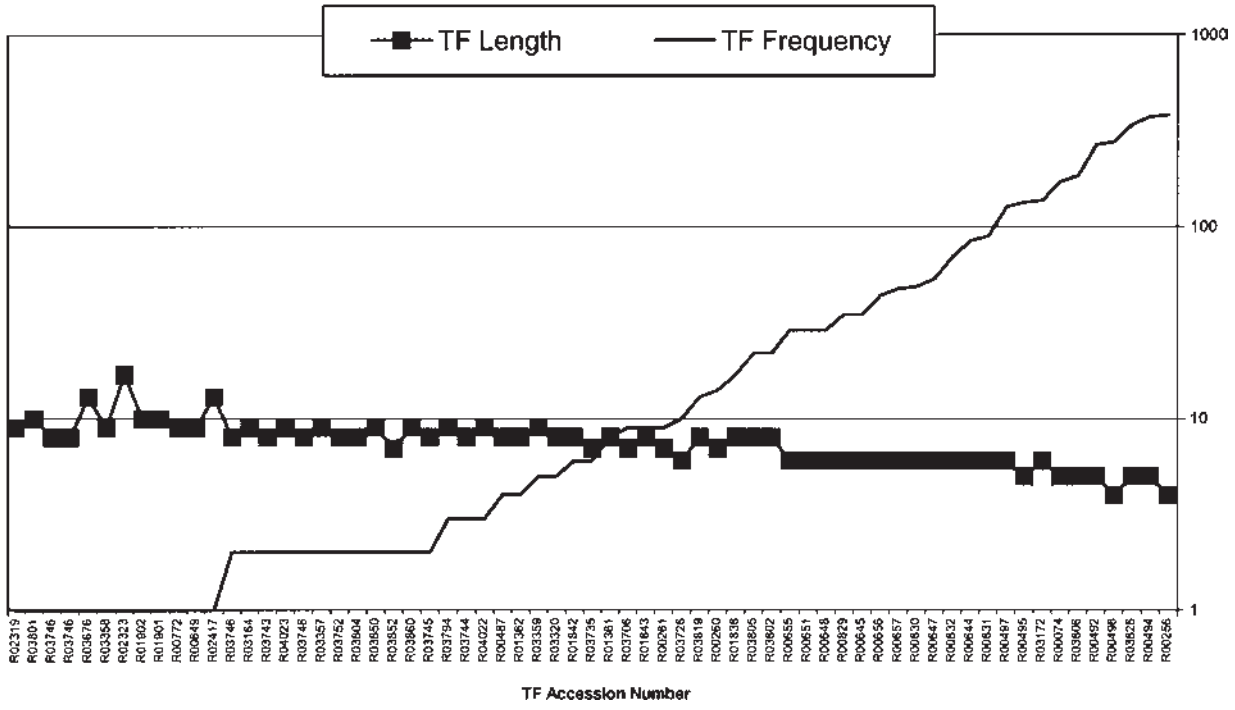

Figure 2 The frequency of occurrence of transcription factors in yeast Chromosome I.

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algorithms that can assist in interpretation and discovery of knowledge contained in the numerous sequence-related databases. Specifically, these tools are expected to focus on completing the transcript map and understanding the functional significance of the sequenced genes. It is anticipated that elements of locus control, like MARs, will be sought during this phase given their key role in genetic processes, and their localization to functional chromatin domains. Thus, a means to model these markers so that they could be placed on the genome sequence map would have significant ramifications from both a biological perspective as well as the development of new gene therapeutics.

Data mining and knowledge discovery techniques can be applied to genomic sequences to detect elements of locus control that impart gene expression in a position-independent copy-number dependent manner. The MARs are one such type of locus control element. Our limited knowledge of MARs has hampered formulating their detection using classical pattern recognition where the existence of lower level constituent elements is used to establish the presence of a higher-level functional block.

The MARs have been experimentally defined for several gene loci, including, the chicken lysozyme gene, human interferon-β gene, human β-globin gene, chicken α-globin gene, p53 and the human protamine gene cluster. Several motifs that characterize MARs have emerged although a MAR consensus sequence is not apparent. The following characteristics of DNA known to be associated with the presence of matrix association regions (summarized from Boulikas 1993; Van Drunen et al. 1999):

The origin of replication (ORI): It has been established that replication is associated with the nuclear matrix, and the ORI share the ATTA, ATTTA, and ATTTTA motifs.

Curved DNA: Curved DNA has been identified at or near several matrix attachment sites and has been involved with DNA-protein interaction, such as recombination, replication and transcription. Optimal curvature is expected for sequences with repeats of the motif, $AAAAn_7AAA > n_7AAAA$ as well as the motif TTTAAA.

Kinked DNA: Kinked DNA is typified by the presence of copies of the dinucleotide TG, CA, or TA that are separated by 2–4 or 9–12 nucleotides. For example, kinked DNA is recognized by the motif TAn_3TGn_3CA, with TA, TG, and CA occurring in any order.

Topoisomerase II sites: It has been shown that Topoisomerase II binding and cleavage sites are also present near the sites of nuclear attachment. Vertebrate and Drosophila's topoisomerase II consensus sequence motifs can be used to identify regions of matrix attachment.

AT-rich sequences: Typically many MARs contain stretches of regularly spaced AT-rich sequences in a periodic manner.

TG-rich sequences: Some T-G rich spans are indicative of MARs. These regions are abundant in the 3'UTR of a number of genes, and may act as recombination signals.

Consensus motif: The sequence TCTTTAATTTCTAAT-ATATTTAGAA defined as the nuclear matrix STAB-1 binding motif.

ATC rule: ATC rule (a stretch of 20 or more occurrences of H, i.e., A or T or C). The ATC rule was used in the analysis of Rice A1-Sh2 region by some researchers. This rule has shown to be an effective indicator of regions with marked helix destabilization potential.

Bipartite signal: A bipartite sequence signature has been reported to be associated with the MARs. This is composed of two degenerate sequences AATAAYAA and AWWRTAANNWWGNNNC within a close proximity.

A-Box, T-Box, etc., and other motifs that have been associated with MARs.

Again, a weight is associated with each motif (and pattern) that is inversely proportional to the probability of its random occurrence. This value can be derived using the base composition of the sequence being analyzed. The search for MARs results from defining a group of patterns that are bonded together in order to form a biologically functional unit as classified by their similar function. After such a grouping, a search for the patterns in a given group can be performed to identify these regions in the query DNA sequence. If a large subset of members of a functionally related group of patterns is found in a specific region of the uncharacterized DNA sequence, one can begin to learn about its function.

It is quite intuitive to consider pattern-cluster density as a property defined along the span of a sequence. A sliding window algorithm can be applied for measuring this value, where the measurements are characterized by the two parameters, W and δ. The cluster-density is measured in a window of size W centered at location x along that sequence. Successive window measurements are carried out by sliding this window in the increments of δ nucleotides. If δ is small, linear interpolation can be used to join the individual window estimates that are gathered at $x, x+\delta, \ldots, x+k\delta$. In this manner, a continuous distribution of the cluster-density is obtained as a function of x.

The task of estimating the density of pattern clusters in each window can be statistically defined as a functional inverse of the probability of rejecting the null hypothesis, that states that the frequency of the patterns observed in a given window is not significantly different from the expected frequencies from a random W nucleotide sequence of the same composition as the sequence being analyzed. The inverse function chosen as, $\rho = -\log(\alpha)$, where the parameter α is the probability of erroneously rejecting H_0. In other words, α represents the probability that the set of patterns observed in a window occurred purely by chance. The value of ρ is computed for both the forward and the reverse DNA strands since we do not know if one strand or

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both strands will be bound, the average of the two values is considered to be the density estimate at a given location.

In order to compute ρ, assume that we are searching for k distinct types of patterns within a given window of the sequence. In general, these patterns are defined as rules $R_1, R_2, \ldots, R_k$. Assume that these probabilities for k patterns are $p_1, p_2, \ldots, p_k$.

Next, a random vector of pattern frequencies, F, is constructed. F is a k-dimensional vector with components, $F = \{x_1, x_2, \ldots, x_k\}$, where each component x_i is a random variable representing the frequency of the pattern R_i in the W base-pair window. The component random variables x_i are assumed to be independently distributed Poisson processes, each with the parameter $\lambda_i = p_i W$. Thus, the joint probability of observing a frequency vector $F_{obs} = \{f_1, f_2, \ldots, f_k\}$ purely by chance is the one-sided integral α of the multivariate Poisson distribution (Mardia et al. 1979) and is utilized to compute the value of ρ or the cluster-density as specified in Eq. (7) below:

$$\rho = \ln\frac{1.0}{\alpha} = -\ln(\alpha)$$

$$= \sum_{i=1}^{k} \lambda_i + \sum_{i=1}^{k} \ln f_i! - \sum_{i=1}^{k} f_i \ln\lambda_i \qquad (7)$$

6 RESULTS

This statistical inference algorithm based on the association of patterns found within the close proximity of a DNA sequence region has been incorporated in the *MAR-Wiz* tool (Singh et al. 1997). A java-enabled version of the tool is available for public access at http: //www.futuresoft.org/MARWiz. A similar algorithm was developed for the analysis of regions of high transcription activity.

MAR-Wiz was originally developed for analyzing sequences from cosmid sized (~40 kb) sequencing projects, the initial backbone of HGP. The program default values of window length (1000), step size (100) and run length (3), i.e., number of concurrent steps were implemented accordingly. For cosmid sized or larger sequences, step size should be maintained at 100 bp, to minimize noise while permitting sufficient discrimination. The predicted locations of regions of matrix attachment correspond well with those experimentally determined. As shown in Figure 3, this strategy was effectively utilized to identify and classify the MARs of the PRM1 → PRM2 → TNP2 locus to the sperm-specific class. It must be emphasized that the predictions of MAR-Wiz can only provide a guide to biological verification.

Figure 4 shows the analysis of Chromosome I for *Saccharomyces cerevisea*. The clustering of motifs with a low likelihood of occurrence by chance within any given region results in a single small region of very high MAR potential. The analysis is performed with and without the repetitive DNA present in the sequence. CENSOR program (Jurka et al. 1996) was utilized for masking out the repetitive DNA stretches.

Figure 5 shows the MAR and the transcription factor analysis of Chromosome I for *S. cerevisea*. A correlation between the high density of transcription factor binding sites and the matrix attachment regions is evident in this plot. This plot will assist in identifying regions further biological investigation.

7 CONCLUSIONS

This chapter presented an overview of the various types of patterns that are observed within the nuclear DNA. The genes in a eucharyotic genome are like a switch and have two potentiative states, i.e., open or closed, with the expression of genes occurring in the open regions. The two potentiative states, ready/open or off/closed, are correlated with the presence or absence of multiple factors interacting with element(s) that are far distal of their respective gene-specific promoters and enhancers at regions of locus control. To date, three classes of elements that act as regions of locus control have been identified. This suggests that gene potentiation may have multiple and/or redundant mechanisms. The classes of

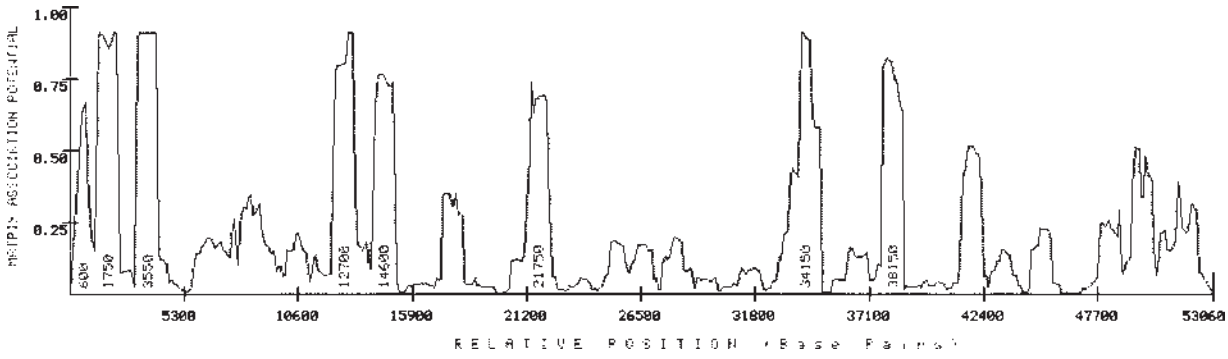

Figure 3 The results of analyzing the human protamine gene cluster using the MAR-Wiz algorithm.

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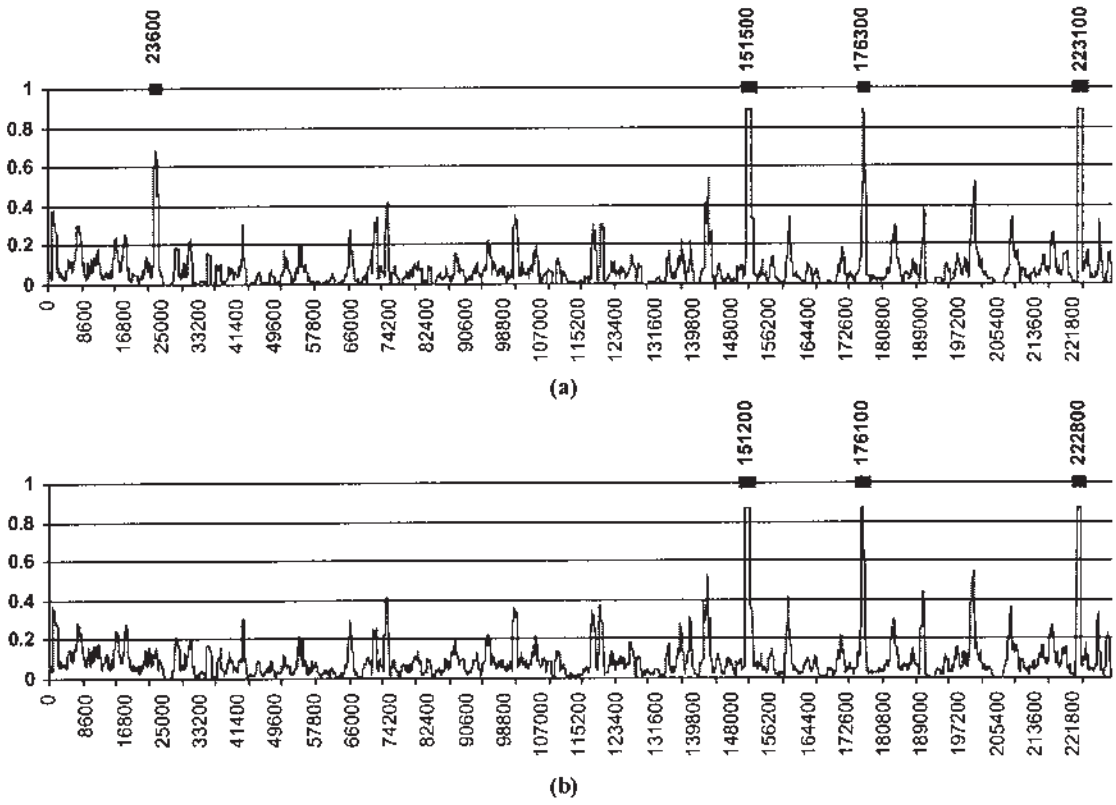

Figure 4 The MAR analysis of Chromosome I: (a) without masking of the repetitive elements and (b) with the repetitive elements masked out. It is evident that one of the MARs in the chromosome occurs within repetitive DNA.

elements are, MARs/SARs, SCSs, and LCRs. These elements provide a dominant chromatin opening function that is an absolute requisite for transcription of this segment of the genome. In this manner phenotype is ultimately defined by gene potentiation.

Out of the approximately 100,000 MARs in a typical mammalian somatic cell, 30,000–40,000 serve as ORI and substantial number appear to be localized towards the centromere and are only used for attachment in somatic cells. The function of the remaining MARs is yet to be established. The observation that MARs most often flank the ends of genic domains encompassing various genic units points to their possible role in gene expression.

A bioinformatics strategy for understanding the co-occurrence of sequence level patterns, with the objective of highlighting the higher level coordination was discussed in this chapter. The biological functionality of some these known lower level patters was discussed.

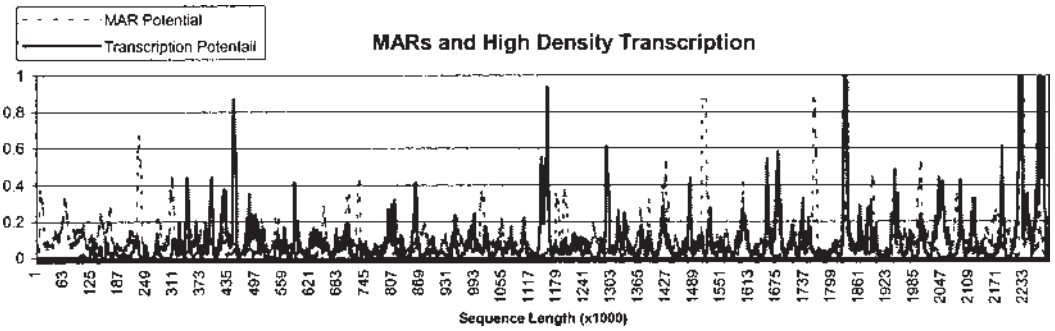

Figure 5 A cumulative analysis of yeast Chromosome I using MAR detection algorithm and isolation of transcription density regions.

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The techniques outlined were applied to the processing of yeast Chromosome I.

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14

DNA Chips and Microarray Analysis—An Overview

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1 INTRODUCTION

DNA microarray technology has given rise to the study of functional genomics (Brown and Botstein 1999; Lockhart and Winzeler 2000). The entire set of genes of an organism can be microarrayed on an area as small as a fingernail and the expression levels of thousands of genes are simultaneously studied in a single experiment. DNA microarray technology allows comparisons of gene expression levels on a genomic scale in all sample combinations. For example, samples derived from normal and diseased tissues, treated and nontreated time courses, and different stages of differentiation or development can be compared. Further computational analysis of microarray data allows the classification of known or unknown genes by their mRNA expression patterns. Global gene expression profiles in cells or tissues will provide us with a better understanding of the molecular basis of phenotype, pathology, or treatment. This review article presents an introduction to the DNA microarray technology.

2 MICROARRAY FABRICATION

On a glass surface, complementary DNAs (cDNAs) or gene-specific oligonucleotides can be spotted. Oligonucleotides can also be synthesized *in situ*.

2.1 cDNA or Pre-Synthesized Oligonucleotide Deposited Arrays

In deposited arrays, which were developed by Pat Brown lab in Stanford University (Schena et al. 1995; the general guide is well described in the web site of http://cmgm.stanford.edu/pbrown/mguide/index.html), a high-speed spotting robot is used to affix PCR-amplified cDNA or synthesized oligonucleotides onto a chemically modified glass slide. Polylysine or aminosilane is commonly used as a substrate for coating the surface of these glass slides. The DNA arrayed slides are then hybridized with fluorescently labeled cDNAs reverse-transcribed from mRNA populations. During this process, the slide is hybridized with two different cDNA samples labeled separately with two distinct fluorescent dyes, such as Cy3 and Cy5 (two-color hybridization). The relative intensities of the two fluorescent dyes within a spot represent the relative mRNA expression levels of the gene. For example, if fluorescent labels Cy3 (green) and Cy5 (red) are used to make each sample's cDNA probe, the expression level of a gene will be displayed as green or red when the gene is differentially expressed, or yellow when the level is the same in both samples (Figure 1).

Mechanical microspotting allows transfer of premade substances onto a solid surface. The recently developed inkjet printing method (Agilent, Palo Alto, CA) produces more uniform spots (Figure 1A), which had been difficult to achieve with available pin spotting techniques.

2.2 *In Situ* Synthesized Oligonucleotide Arrays

This method was developed by Fodor et al. (1991) and it is now referred to as the Affymetrix GeneChip system (Affymetrix Inc., Santa Clara, CA). In these oligonucleotide arrays, DNA oligonucleotides are synthesized *in situ* onto the DNA chip using photolabile protecting groups and photolithographic masks to add the selective sequences of nucleotides (Lipshutz et al. 1999). The recent version of GeneChip contains 400,000 of 25-base-pair (bp) oligonucleotides where twenty different oligonucleotide pairs represent one gene. A pair consists of a "perfect-match" and a

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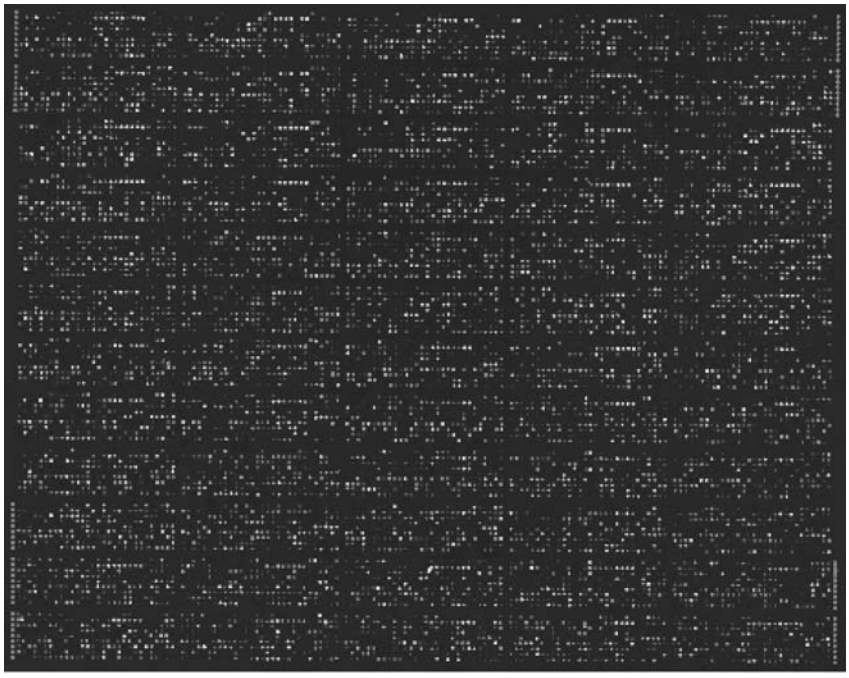
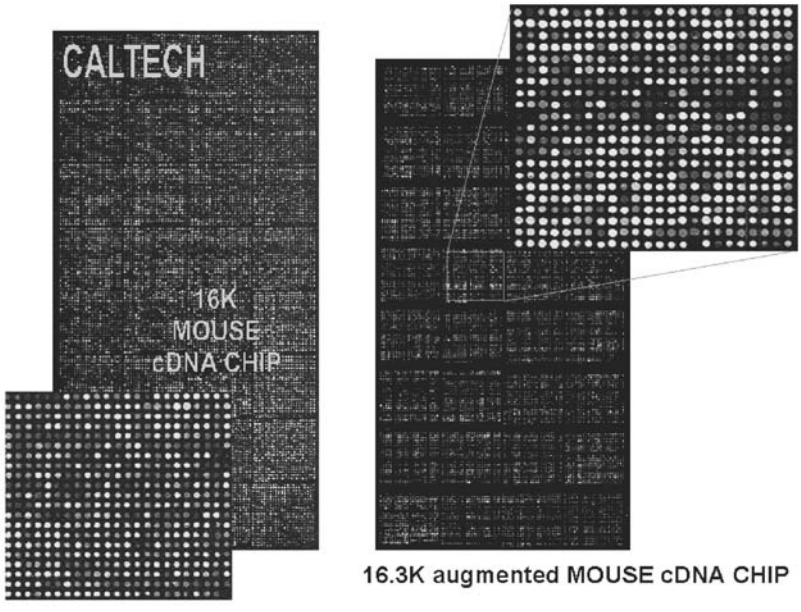


(a)



(b)

Figure 1 Deposited cDNA microarrays cohybridized with fluorescently labeled reference (Cy3) and test (Cy5) samples. The hybridization output is measured by the relative intensities of the two colors, red (Cy5) and green (Cy3). Yellow spots indicate equal amounts of expressed mRNA from each sample, red indicating higher expression in the test sample and green lower expression. (a) Inkjet spotted cDNA array. (b) Pin spotted cDNA array.

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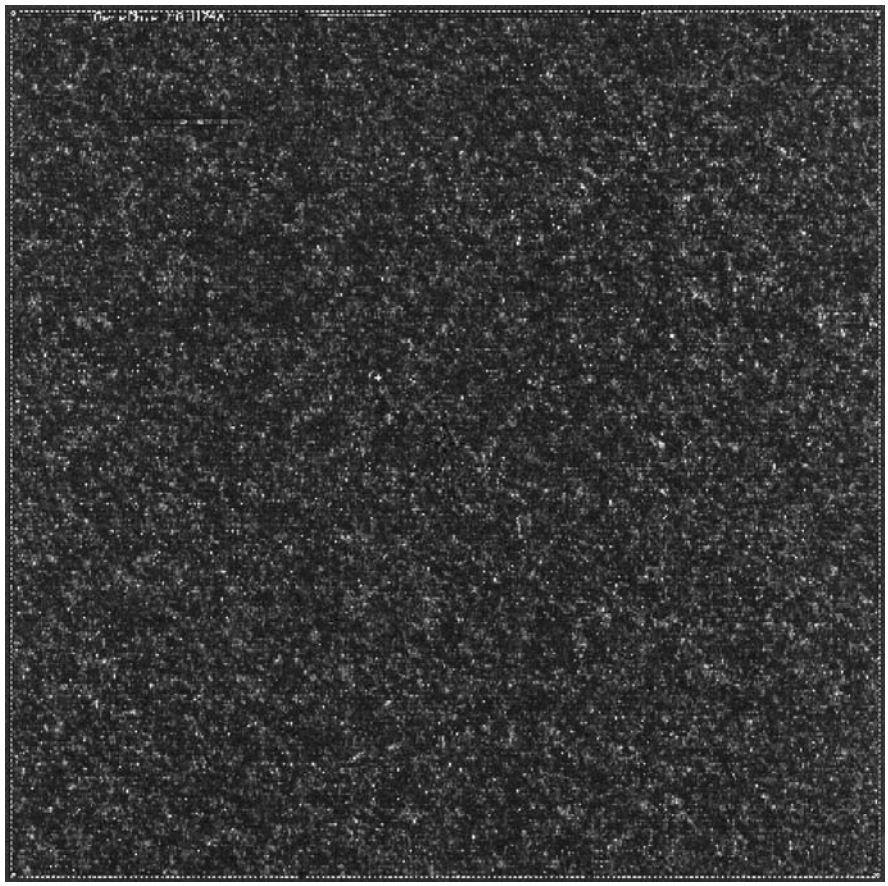

Figure 2 Affymetrix GeneChip image.

"mismatch" oligonucleotide, in which the 13th nucleotide is mutated. The "perfect match" signals are subtracted by "mismatch" signals, and the net values are used for the comparisons. In the Affymetrix GeneChip system, in contrast to the cDNA or pre-synthesized oligonucleotide deposited arrays, only one sample is hybridized onto one array and comparisons can be made among multiple arrays computationally (one-color hybridization) (Figure 2).

2.3 cDNA vs. Oligonucleotide as a Target DNA

For reference, the term "probe" refers to the fluorescently labeled DNAs or RNAs and the term "target" refers to the DNAs on the slide. The definitions are reversed in some articles, especially when the Affymetrix GeneChip system is mentioned.

The oligonucleotide targets require only the sequence information of genes, and thereby can maximally exploit the genome sequences of the organism. The precise fold changes from cDNA or oligonucleotide microarrays are not identical, but the general trends of changes and the fold differences are similar to each other when the same genes are compared in both array experiments. In oligonucleotide arrays, the design of oligonucleotides is very critical to the results and they should be designed very carefully. In cDNA arrays, there may be potentially confounding effects of cross-hybridization due to sequence homologies among members of a gene family. When oligonucleotides are used, they can be specifically designed to differentiate between highly homologous members of a gene family as well as alternatively spliced forms of the same gene (exon-specific). Oligonucleotide arrays can also be designed to allow detection of mutations and single nucleotide polymorphisms.

3 HYBRIDIZATION

In two-color hybridization, fluorescently labeled cDNA probes, directly reverse-transcribed from two separate mRNA populations, are hybridized onto the target DNA fixed on the slide. One cDNA sample is labeled with Cy3 fluorescent dye, and the other sample with Cy5. After hybridization and washing on a single array, the relative level in mRNA transcript for each gene is measured by the Cy5/Cy3 color ratio using a confocal laser scanner.

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3.1 Preparing Probes

3.1.1 Labeling from Total RNA or Poly(A) mRNA

Total RNA or poly(A) mRNA can be used as a template for microarray analysis. An array generally requires 2–200 μg of total RNA or 0.2–2 μg of poly(A) mRNA as a starting material. These values are adjusted depending on the number of elements, area of hybridization, method of labeling, etc. When poly(A) RNAs are reverse-transcribed, Cy3- or Cy5-linked dNTP is added to generate the final cDNA probe.

A minimum of 0.2 μg poly(A) mRNA or 5 μg total RNA is recommended to start the Affymetrix cDNA synthesis protocol. Basically, the Affymetrix system uses *in vitro* transcription after making double stranded cDNA from poly(A) RNA. During *in vitro* transcription of the complimentary RNA (or linear amplification: details in 3.1.2.), biotin-labeled NTPs are incorporated, probes are hybridized onto the GeneChip, and Streptavidin-Phycoerythrin is used to stain the biotin-bound targets. Phycoerythrin is detected in the wavelength of 570 nm by an appropriate scanner.

3.1.2 Amplification

Direct labeling of cDNA microarray usually requires about 100 μg of total RNA or 1 μg of mRNA to determine an expression profile. This becomes a problem when only a small amount of RNA is available for analysis. In this case, optimized linear amplification methods can be performed to increase the number of transcript messages (Figure 3). One example of this method is using a primer containing the T7 promoter sequence fused to an oligo-dT sequence to generate double strand cDNAs from the template mRNA. Antisense RNAs (aRNA) are then linearly amplified from cDNAs using T7 RNA polymerase. A portion of the amplified aRNAs is now reverse transcribed in the presence of Cy-3-dNTP or Cy-5-dNTP to generate fluorescently labeled cDNAs. The expression results obtained from linear amplified aRNA are comparable to those from the original mRNA.

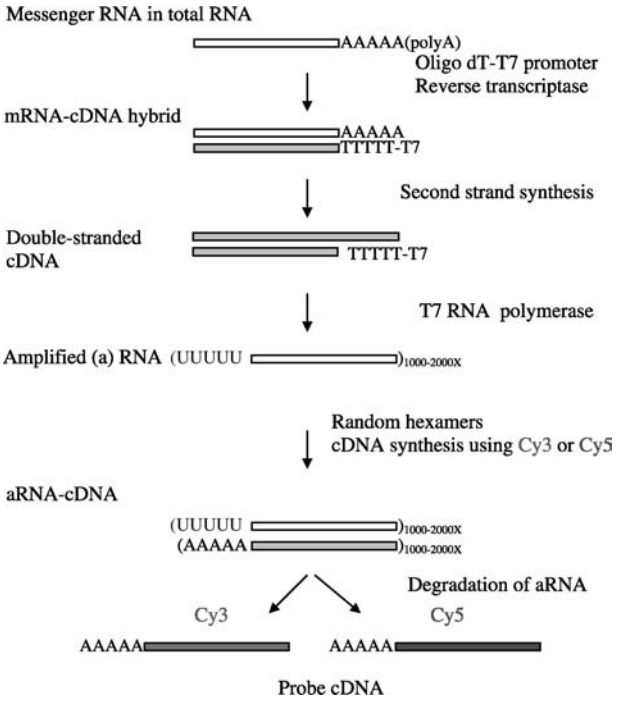

Figure 3 Linear amplification method.

3.2 Laser Capture Microdissection

Integration of laser capture microdissection to microarray technology (Luo et al. 1999) can potentially provide substantially higher homogeneity in samples by determining with microscopic visualization. It is very useful to define the gene expression profile within a small group of a specific cell population. A cell typically produces 10–30 pg of total RNA. An amplification technique is usually needed to generate a sufficient amount of probe from small but homogeneous cell populations for microarray hybridizations. Faithfully amplified cellular RNAs from a few (or even single) cells isolated by laser capture microdissection will reach the ultimate sensitivity of the smallest functional tissue unit. This will eventually lead to a cellular-level molecular understanding of the functional processes.

3.3 Scanning

Hybridized slides are scanned with a confocal laser scanner, such as GenePix 4000A scanner (Axon Instruments, Union City, CA), ScanArray (GSI Lumonics, Farmington Hills, MI), Agilent Scanner (Agilent, Palo Alto, CA), etc. The majority of the confocal laser microarray scanners use photomultiplier tubes (PMTs) as detectors. The PMTs can detect fluorescence intensities in the visible wavelength range. By varying the control voltage, the sensitivity of the detector can be modified easily. The acquired images are analyzed with one of the available softwares, such as GenePix Pro3.0 (Axon) and Image Analysis (Agilent).

4 DATA ANALYSIS

4.1 Normalization

4.1.1 Total Intensity Normalization

The total intensity normalization method stands on the assumption that the total quantities of messages from both samples are the same. Under this assumption, a normalization factor can be calculated from the total integrated intensity (in one color hybridization, for example, in

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Affymetrix GeneChip system) or from the total average fold difference of the Cy3 and Cy5 channels (in two color hybridization, for example, in deposited cDNA arrays) for all the elements in one array. This normalization factor is then used to adjust the scale or fold for each gene in the array.

4.1.2 Regression Normalization

In a scatter plot of both channels in the two-color hybridization, the genes would scatter along a diagonal straight line when closely related two samples are compared. Normalization of this data can be performed by calculating the best-fit slope and by applying the regression to adjust the levels of all the genes. Adjustment using local regression is more suitable in cases where the fold differences are nonlinear.

4.1.3 Normalization Using Ratio Statistics

This method assumes that a subset of genes, referred to as "housekeeping genes," do not change their profiles throughout the experiments. The normalization factor calculated from this subset of housekeeping genes is used to adjust experimental variability in the samples being compared. Alternatively, a set of exogenous controls can be spiked onto the arrays and mRNAs from the set are equally added into the initial RNA samples before labeling. The average expression ratio from these controls should be equal to one and this factor is used to normalize the data to identify differentially expressed genes.

4.1.4 Universal Standard

Great advantages arise in the scientific community when research data is shared between laboratories and experiments. Unfortunately, microarray data is not easily shared due to the variation of standards among experiments. The need for researchers to agree on one particular standard, referred to as the universal standard, is very difficult to achieve. Thus, ongoing efforts to find a common standard sample for all experiments are in progress to facilitate widespread data sharing.

There are also some critical caveats in using a universal standard: (a) When everyone attempts to prepare a large batch of the reference sufficient for the entire project, the reproducibility among the standards cannot be assured. (b) Standards as denominators below the threshold of accurate measurement must be avoided. (c) An alternative, much discussed in the array community, is a pool of about 10 cell lines. The universal human or mouse reference RNA isolated from 10 (human) or 11 (mouse) cell lines representing different tissues is available from Stratagene (La Jolla, CA). The idea is that this will cover most genes, and that with fresh culture, although there might be differences in some cell lines, the effects would be smoothed out over the entire group.

Still there may be some problems associated with universal standards. For example, you cannot hope to obtain better data than the system will produce under idealized conditions. Whenever possible, the use of a direct comparison—the same cells split into two different conditions and then co-hybridized—will yield the most accurate results, particularly for small induction/repression levels. However, a universal standard, if possible, is the best way overall to ensure consistency for large data sets.

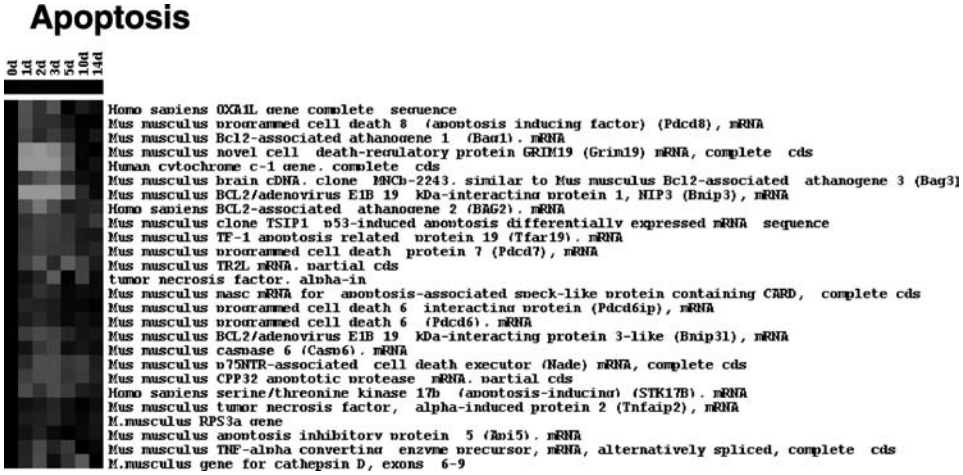

Figure 4 Hierarchical clustering of gene expression profiles: The column lists the time point studied. The row corresponds to individual gene surveyed. Upregulated genes appear in red, and downregulated genes appear in green, with the relative $\log_2$ ratio reflected by the intensity of the color.

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4.2 Clustering

DNA microarray experiments generate unprecedented quantities of genome-wide data which can greatly overwhelm biologists. To extract useful information from expression profiles, computational tools that cluster and display data can be used. Although there are many ways to analyze gene expression data, hierarchical clustering (Eisen et al. 1998) and self-organizing map clustering (Tamayo et al. 1999) have been widely used to display the data.

Hierarchical clustering is simple and the results are easily visualized (Figure 4). In hierarchical clustering, the distances between genes are calculated for all of the genes based on their expression pattern and the closer genes are merged to produce a cluster. The distances between these small clusters are calculated to produce a new cluster.

Self-organizing map (SOM) clustering assigns genes to a series of groups on the basis of expression pattern similarities. Random vectors are constructed for each group and a gene is assigned to the closest vector.

5 APPLICATIONS OF MICROARRAYS

Traditional molecular research tools for gene expression study are limited to a small group of genes at a time. Recent advances in microarray field have enabled the study of large numbers of genes in a single experiment. DNA microarrays not only detect global changes of gene expression, but also have many other potential applications including the identification of gene copies in a genome (Pinkel et al. 1998), mutation and polymorphism detection (Wang et al. 1998), sequencing, diagnostic tools for diseases, and drug discovery.

6 BEYOND THE DNA MICROARRAYS

The question in dealing with the current humongous amount of microarray data is whether we are ready to decode these messages. Very often, the array data might not apply well to our previously accumulated knowledge. This is due to the fact that data obtained from expression profiling are too complex to interpret and for a long time we have been used to studying one gene or one system at a time. Although the array data on a genomic scale is significantly insightful in understanding the mechanisms in biological systems, mRNA profiling provides us with only the levels of mRNA messages. The problem of biological interpretation of gene expression data occurs when cellular events are mediated in protein levels. In addition, the current array data include the transcriptional behaviors of a large portion of yet-uncharacterized genes.

Recently NCBI has launched the Gene Expression Omnibus (GEO) site (http://www.ncbi.nlm.nih.gov/geo/), allowing public access to a broad range of gene expression data. GEO is not only a gene expression data repository but also an online resource for the retrieval of gene expression data from any organism and among laboratories. The meaningful use of these and other array data will require systematic computational analyses and cross-comparisons with other data sets obtained from different experimental systems, such as two-dimensional gel electrophoresis of proteins, yeast two-hybrid system, and other conventional molecular biological tools.

7 CONCLUSIONS

By the time this article is published, it is expected that the entire human or mouse genes will be available on a chip. Since DNA microarrays have the possibility of incorporating thousands of genes, it would not be impossible to scan the whole genome of a particular organism in one experiment. Thus, these techniques will allow the complete comparison of almost all transcribed genes' expression levels.

The influence of microarray technology has been powerful in both basic and applied biology. Unmanageable amounts of microarray data have out-paced and even stimulated the development of a new science area, bioinformatics. One important goal of computational analysis is to extract clues from microarray data and translate the information into biological understanding. Systematic analysis of microarray data will yield insight into molecular biological processes and functions of thousands of gene products in parallel. This approach allows for improved understandings in cellular signaling, disease classification, diagnosis, prognosis, and drug design. To pinpoint specific genes as research targets, such as drug targets, traditional methods are still required. What microarrays do best is high-throughput screening.

The future of plant microarrays will diverge from *Arabidopsis* (Finkelstei et al. 2002) to agriculturally important plants, such as maize, rice, soybean, etc. When the genome sequencing is completed, full genome microarrays can also be constructed. By using microarrays, disease resistance, cold or drought response, and product yield can be explored on a genomic scale and comprehensive dynamic view of what happens in the molecular level.

Proteome technologies for monitoring changes in protein abundance and protein modification are important because the correlation between gene and protein expression is variable, and the post-translational protein modifications are responsible for realizing the signaling and information processing. Tissue microarrays (Kononen et al. 1998) and protein microarrays (MacBeath and Schreiber 2000; Zhu et al. 2001) have been developed where samples from up to hundreds of tissues or proteins are analyzed simultaneously on one glass slide.

As with DNA microarray, orchestrated and genome-wide gene expression studies can be efficiently carried out, and these explorations will provide clues for new hypotheses. Microarray technologies will accelerate scientific discoveries and become one of the key technologies in biology.

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15

Signal Transduction in Fungi: Signaling Cascades Regulating Virulence in Filamentous Fungi

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1 INTRODUCTION

Recognition of environmental stimuli plays a fundamental role for eukaryotic cells. Many different signals are received at the surface of cells and submitted by transmembrane signaling pathways to elicit specific responses such as the induction of gene transcription, protein phosphorylation, or cytoskeleton reorganization. The movement of signals can be simple, such as those associated with receptor molecules that constitute channels which upon ligand interaction, allow the signals to be passed in the form of small ion movements, either into or out of the cell. These ion movements result in changes in the electrical potential of the cells and these in turn propagate the signal along the cell. More complex signal transduction involves the coupling of ligand–receptor interactions to many intracellular events which include phosphorylations by different kinases. The receptors used are of different types: e.g., G-protein-coupled receptors, His–Asp phosphorelay sensors, etc. The eventual outcome is an alternation in cellular activity and changes in the program of genes expressed within the responding cells that results in a cellular response to the external environment. Eukaryotic cells predominantly use serine, threonine, and tyrosine phosphorylation in various intracellular signal transduction pathways, among which the MAPK cascade module is a key element in mediating the transduction to the nucleus of many signals generated at the cell surface (Banuett 1998). Fungal cells can also sense many different chemical and physical stimuli such as nutrients, osmolarity, pH, light, or surface hydrophobicity. Furthermore, fungi recognize the presence of mating partners by pheromones and pathogenic fungi respond to signals from their respective hosts. Besides the model organism *Saccharomyces cerevisiae* whose signaling cascades are quite well characterized (e.g., Banuett 1998; Gustin et al. 1998; Hunter and Plowman 1997; Thevelein 1994), the information about signal transduction pathways in filamentous fungi is fragmentary. Even though a significant percentage of the fungal genome encodes phosphorylation-related proteins, little is understood regarding their function, the interacting molecules, and pathways in which they are involved. Investigation of the signal transduction cascades that regulate fungal development and virulence and the related signaling pathways that operate in yeast, human pathogens, plant pathogens, and in model filamentous fungi reveal a high degree of conservation.

Among the best investigated fungal systems, where at least some cascades are partially characterized are the plant pathogenic fungi as *Magnaporthe grisea*, *Ustilago maydis*, and *Cryphoncetria parasitica*. These fungal pathogens have evolved strategies to recognize suitable hosts, penetrate and invade the host tissue, overcome host defenses, and optimize growth in the host. To perform these tasks, the fungus must receive chemical and physical signals from its host and respond with appropriate metabolic and morphogenetic changes. Many of these responses require the expression of specific genes and are dependent on conserved signal transduction pathways involving components such as G proteins (Bölker 1998), cAMP signaling (Lee and Dean 1993; Mitchell and Dean 1995), and MAPK cascades (Xu and Hamer 1996; Xu et al. 1998). Because of the multiplicity and variety of components and cascades involved in fungal signal transduction pathways, the following chapter will mainly focus on signaling in selected plant pathogenic filamentous fungi and will compare this to the respective cascades known

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from yeast and the isolated information available on other fungal species.

2 SIGNAL TRANSDUCING RECEPTORS

Cells can recognize external stimuli and react to them by producing intracellular responses. Membrane-impermeable signal molecules are recognized by receptors localized on the plasma membrane. Binding of a ligand results in a conformational change of the receptor and thereby in the stimulation of an intrinsic enzymatic activity or the modulation of a transducing protein or ion channel (Schenk and Snaar-Jagalska 1999).

Amongst the different families of known integral receptors, the G protein-coupled receptors (GPCRs) comprise the largest family, and these mediate the actions of extracellular signals as diverse as light, odors, hormones, pheromones, calcium ions, and neurotransmitters. G protein-coupled receptors have been identified in organisms as evolutionary divergent as yeast and man, and nearly all bear detectable sequence similarity with one another (Dohlman et al. 1991; Hur and Kim 2002).

2.1 G Protein Coupled Receptors

G protein coupled receptors share a common domain structure containing seven stretches of hydrophobic amino acids spanning the cytoplasmic membrane with the N-terminus located outside of the cell and the C-terminus within the cytoplasm (Dohlman et al. 1991). Ligand binding to the receptor is characterized by a conformational change that leads to the release of the G protein and the exchange of GDP for GTP on the Gα subunit. The GTP-bound α subunit dissociates from its βγ subunit partner, and both signaling units can regulate the activities of downstream effectors such as adenylate cyclases and phospholipases, stimulate mitogen-activated protein kinases (MAPKs), or directly regulate ion channels (Birnbaumer 1992; Gutkind 1998). Pheromone receptors belonging to the group of GPCRs have been characterized in several fungi including *S. cerevisae* (Hagen et al. 1986; Nakayama et al. 1985), *Schizophyllum commune* (Fowler et al. 1999; Hegner et al. 1999), *Coprinus cinereus* (Olesnicky et al. 1999), *U. maydis* (Bölker et al. 1992), *Ustiligo hordei* (Anderson et al. 1999), *Neurospora crassa* (Pöggeler and Kück 2001), and *Sordaria macrospora* (Pöggeler and Kück 2001).

In addition to pheromones, nutrients have recently also been shown to be recognized by a G protein-coupled receptor (Gpr1p) in *S. cerevisiae*, where Gpr1p and Gpa2p (G protein α-subunit) are part of a G protein-coupled sensing system that activates cAMP-regulated pathways in response to levels of extracellular glucose (Forsberg and Ljungdahl 2001; Lorenz et al. 2001; Versele et al. 2001; Xue et al. 1998). Gpr1p–Gpa2p related sensors also exist in other fungi, such as *Schizosaccharomyces pombe* where *GIT3* encodes a GPCR homologous to Gpr1p (Welton and Hoffman 2000).

G protein-coupled receptors (e.g., β_2 adrenergic, muscarinic acetylcholine, and somatostatin receptors) expressed in *S. cerevisiae* or *S. pombe* show membrane localization and allow antagonist and/or agonist binding (Ficca et al. 1995; Huang et al. 1992; King et al. 1990; Kong et al. 2002; Price et al. 1995). Furthermore, the rat somatostatin receptor SSTR2 and *Schizophyllum* receptors have been shown to couple with the yeast G protein and activate the yeast pheromone-response pathway (Fowler et al. 1999; Price et al. 1995). These experiments show that considerable flexibility exists in the interactions of the GPCRs and yeast Gα proteins and that this flexibility may serve to integrate signals from several sources to a single transduction pathway and/or to promote activation of multiple signal transduction pathways by a single receptor.

The mechanism by which GPCRs translate extracellular signals into cellular changes was initially regarded as a simple linear model where activation of the receptor by agonist binding leads to dissociation of the G protein into its α and βγ subunits which can then activate or inhibit downstream effector molecules. However, the discovery of the family of regulators of G protein signaling (RGS) proteins—acting as negative regulators of G protein signaling—gave a more complex dimension to the mechanism of GPCR pathways [reviewed by Brady and Limbird (2002) and De Vries et al. (2000)]. The *Aspergillus nidulans* FlbA protein that is involved in sporulation and sterigmatocystin production, has been found to harbor a 120-amino acid C-terminal RGS domain (Hicks et al. 1997).

2.2 Receptor-Independent Activators of G Protein Signaling Pathways

Several reports from mammalian systems and from *Dictyostelium* indicate alternative modes of stimulus input to heterotrimeric G proteins, and these do not require direct interaction of the G protein with the seven-membrane span receptor itself (Brzostowski and Kimmel 2001; Cismowski et al. 1999; 2001; Takesono et al. 1999). Takesono et al. (1999) applied an expression cloning system based upon the pheromone response pathway in *S. cerevisiae* to detect mammalian activators of G protein signaling (AGS) in the absence of a GPCR. AGS1, a human Ras-related G protein which functions by facilitating GTP exchange on the heterotrimeric Gα, and AGS 2 and AGS 3, which may serve as alternative binding partners for the G protein subunits, have been identified (Cismowski et al. 1999; 2001; Takesono et al. 1999).

3 HETEROTRIMERIC G PROTEINS IN FUNGAL SIGNAL TRANSDUCTION

3.1 Signaling via Gα Subunits

In contrast to GPCRs where only a few genes that encode these receptors have been isolated from filamentous fungi,

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much more information is available on fungal G proteins. Gα subunits have been isolated from several fungal organisms, most of them by DNA hybridization with heterologous probes or by PCR with degenerate primers deduced from highly conserved regions of the Gα sequences. They can be classified in three major subgroups following a phylogenetic tree generated by multiple alignment where the protein sequences within each subgroup are more closely related to each other than the sequences of different G proteins from a single organism (Bölker 1998):

(a) Members of subgroup I are homologs of the mammalian $G\alpha_i$ subunits that inhibit adenylate cyclase (Turner and Borkovich 1993) evidenced by the presence of both a potential myristoylation site (MGXXXS) at the N-terminus and the consensus site CXXX for ADP ribosylation by cholera toxin (Simon et al. 1991) at the C-terminus. For some members of this group there is genetic evidence that they can lower cAMP levels although the mechanism of this is still unknown (Bölker 1998). Examples of Subgroup I proteins are Gpa1p from *S. cerevisiae* (Miyajima et al. 1987), Gna-1 from *N. crassa* (Turner and Borkovich 1993), FadA from *A. nidulans* (Yu et al. 1996), MagB from *M. grisea* (Liu and Dean 1997), Gpa1 from *U. maydis* (Regenfelder et al. 1997), Cpg-1 from *C. parasitica* (Choi et al. 1995) and Bcg1 from *Botrytis cinerea* (Gronover et al. 2001). (b) Members of subgroup II lack the consensus site for pertussis toxin dependent ribosylation. The direct effectors for members of this fungal G protein subfamily still remain to be identified and a biological function or a distinct phenotype has been observed for only a few of them. Members of this subgroup include Gpa1 from *S. pombe* (Obara et al. 1991), MagC from *M. grisea* (Liu and Dean 1997), Gpa2 from *U. maydis* (Regenfelder et al. 1997) and Bcg2 from *B. cinerea* (Gronover et al. 2001). Gna-2 was isolated from *N. crassa*, but the Δ*gna-2* strain does not possess any obvious phenotypes. However Δ*gna-1* Δ*gna-2* mutants exhibit more severe defects in processes noted for the Δ*gna-1* mutants, and this leads to the assumption that *gna-1* and *gna-2* have overlapping functions and possibly constitute a gene family (Baasiri et al. 1997). (c) Members of fungal Gα protein subgroup III have been implicated as a positive influence on the internal cAMP level, and include Gpa2p from *S. cerevisiae* (Nakafuku et al. 1988), MagA from *M. grisea* (Liu and Dean 1997), Gpa3 from *U. maydis* (Regenfelder et al. 1997), Fil1 from *U. hordei* (Lichter and Mills 1997), Cpg-2 from *C. parasitica* (Choi et al. 1995), and Gna-3 from *N. crassa* (Kays et al. 2000). Therefore, members of this subfamily have been tentatively assigned as adenylyl cyclase stimulating fungal $G\alpha_s$ subunits, analogous to the mammalian $G\alpha_s$ family (Bölker 1998).

It seems that most fungal species possess Gα subunits of each class. Nevertheless, sequencing of the entire genome of *S. cerevisiae* has revealed that *GPA1* and *GPA2* are the only two Gα subunit genes in this organism (Lorenz and Heitman 1997). On the other hand, in *U. maydis* a fourth Gα subunit, Gpa4 has been identified and shown to be larger than all other fungal Gα proteins (Regenfelder et al. 1997).

Targeted gene disruptions have been generated for several fungal Gα subunits in order to study the biological functions of the respective signaling pathways. For a number of pathogenic fungi it has been found that Gα proteins play pivotal roles in the recognition process, virulence and virulence-dependent developmental processes such as the formation of infection structures. The requirement for Gα proteins and their involvement in virulence and pathogenicity has been demonstrated for *magB* from *M. grisea* (while inactivation of *magA* and *magC* affected neither pathogenic development nor vegetative growth; Liu and Dean 1997) *gpa3* from *U. maydis* (Regenfelder et al. 1997), and *cpg-1* from *C. parasitica*. *cpg-1* was further shown to regulate the induction of a *C. parasitica* cellobiohydrolase I, a putative cell-wall degrading enzyme (Chen et al. 1996; Wang and Nuss 1995). In *B. cinerea*, the expression of both Gα subunit-encoding genes was detected in early infection stages and both genes seem to be involved in virulence. *bcg1* null mutants do not secrete extracellular proteases and show clearly reduced pathogenicity on bean and tomato, while *bcg2* mutants are reduced in pathogenicity, albeit only slightly (Gronover et al. 2001).

3.2 Gβ Subunits and Other Components Involved in G Protein Signaling

Cryphoncetria parasitica is the first filamentous fungus from which both a gene for a β subunit (*cpgb-1*) of heterotrimeric G proteins and a gene for another component of G protein signaling (*bdm-1*) have been isolated. Disruption of the *cpgb-1* gene resulted in a phenotype with an increased vegetative growth but reduced virulence (Kasahara and Nuss 1997), whereas *bdm-1* was shown to be either required for or to facilitate Gβ function and, therefore, also has a role in the regulation of virulence (Kasahara et al. 2000). Gβ subunits have further been characterized from *Cryptococcus neoformans*, where *gpb1* is involved in mating and haploid fruiting (Wang et al. 2000), *A. nidulans*, where the *sfaD* gene is required for normal growth and repression of sporulation (Rosen et al. 1999) and *N. crassa* (Ivey et al. 1999).

As already mentioned previously, the sequence of the *S. cerevisiae* genome revealed only two genes encoding Gα homologs (*GPA1* and *GPA2*) of heterotrimeric G proteins but eight candidate Gβ genes and three candidate Gγ genes were also identified (Lorenz and Heitman 1997).

In *S. cerevisiae*, Gβγ activates the pheromone-stimulated MAP kinase pathway and it is known to bind to the N-terminal region of the scaffold protein Ste5p (Whiteway et al. 1995). Furthermore, yeast Gβγ was also shown to activate Cdc24p, the exchange factor for the Rho-type GTPase Cdc42p, and to bind to other members of the Rho family of GTPases as well as to the small G protein Arfp (ADP-ribosylation factor) (Hamm 1998). Recently, Klein et al. (2000) fused yeast *STE4* (encoding the Gβ subunit) and *GPA1* (encoding the Gα subunit) genes and showed that

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the resulting nondissociable Ste4p–Gpa1p fusion protein was fully active in transducing the pheromone signal. Thus, in this system, subunit dissociation is not required for signal transduction.

Adenylyl cyclase and MAP kinase cascades are potential targets of G protein signaling (Figure 1) and some fungal Gα subunits have been shown to be involved in both signaling pathways (Bölker 1998). Nevertheless, for many of these signal transducers, the specific pathways in which they participate are still unknown.

4 CAMP SIGNALING IN FUNGI

cAMP signaling is involved in a variety of processes in fungi including the control of differentiation, mating processes, virulence, monitoring of the nutritional status, and stress. The cAMP pathway (Figure 2) also influences transcription and cell cycle progression (Kronstad et al. 1998). In fungal cells, synthesis of cAMP is regulated by a membrane-associated adenylate cyclase and degradation is regulated by a cAMP-specific phosphodiesterase. In most fungi, the activity of adenylate cyclase, which synthesizes the intracellular messenger cAMP, has been shown to be regulated by

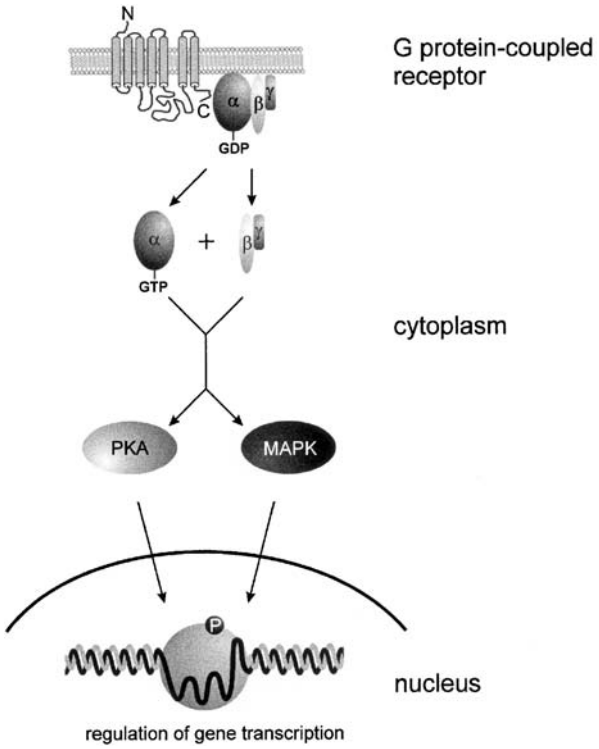

Figure 1 A model of the regulation of gene transcription by cAMP and MAPK pathways as targets of G protein signaling. The GPCR transmits the extracellular signal via the Gα or Gβγ subunits of heterotrimeric G proteins.

α-subunits of heterotrimeric G-proteins. Most of the effects of cAMP in eukaryotes occur via the stimulation of cAMP-dependent protein kinases (PKA) which consist of two regulatory and two catalytic subunits (Dickman and Yarden 1999).

Genes encoding the catalytic subunit and/or regulatory subunit of PKA have been cloned from a number of filamentous fungi including *M. grisea* (Mitchell and Dean 1995), *N. crassa* (Bruno et al. 1996), *Aspergillus niger* (Bencina et al. 1997), *U. maydis* (Dürrenberger et al. 1998; Gold et al. 1997), and *Colletotrichum trifolii* (Yang and Dickman 1999). In *S. cerevisiae* three PKAC (catalytic subunit of PKA) encoding genes (*TPK1*, *TPK2*, and *TPK3*) have been found and it has been demonstrated that each catalytic subunit has a distinctive function for pseudohyphal growth, iron uptake, and respiration, respectively (Robertson and Fink 1998; Robertson et al. 2000).

In plant pathogenic fungi, growth, morphogenesis, and virulence are known to involve functional PKA, and the disruption of the *cpkA* gene (encoding the PKA catalytic subunit) in *M. grisea* led to mutants delayed in appressorium formation and unable to successfully penetrate host tissues. *CpkA* mutants are, however, still able to grow invasively once host tissue barriers have been overcome such as in infections initiated by abrasion of the leaf surface or injection (Xu et al. 1997). Furthermore, *mac1* (*M. grisea* adenylate cyclase gene) disruption mutants were also unable to form appressoria and were reduced in vegetative growth and conidiation (Adachi and Hamer 1998; Choi and Dean 1997). Subsequent studies on this organism revealed that appressorium formation is dependent on both cAMP and MAP kinase Pmk1 (see later).

In *U. maydis* (Figure 2), adenylate cyclase (Uac1), cAMP, and the regulatory subunit of PKA (Ubc1) regulate dimorphic switching (filamentation) during the mating response as well as cytokinesis, bud site selection, and gall formation (Gold et al. 1997). Two genes, *adr1* and *uka1* that encode isoforms of the PKA catalytic subunit have been identified. Dürrenberger et al. (1998) demonstrated the requirement of *adr1* for virulence, whereas no obvious phenotype was observed for the *uka1* mutant. In summary, the cAMP cascade regulates mating-type gene expression and pathogenicity in *U. maydis*. Additionally, it was shown that signaling by the cAMP and MAP kinase (see later) pathways is coordinated and that the activity of the transcription factor pfr1 (pheromone response factor 1) is regulated by cAMP as well as MAP kinase signaling, leading to the assumption that Prf1 functions as an integrator between both pathways (D'Souza and Heitman 2001; Kahmann et al. 1999).

Exogenous cAMP also partially restored wild-type phenotypes in *M. grisea* and *U. maydis* Gα or adenylate cyclase mutants, suggesting that PKA is the downstream target of G protein and/or adenylate cyclase (Choi and Dean 1997; Gold et al. 1994; Liu and Dean 1997; Regenfelder et al. 1997). In *C. parasitica* it was found that defects in cAMP signaling impair the ability of the fungus to cause chestnut blight disease (Gao and Nuss 1996) and in *C. trifolii*, PKA-deficient strains were unable to infect intact host plants

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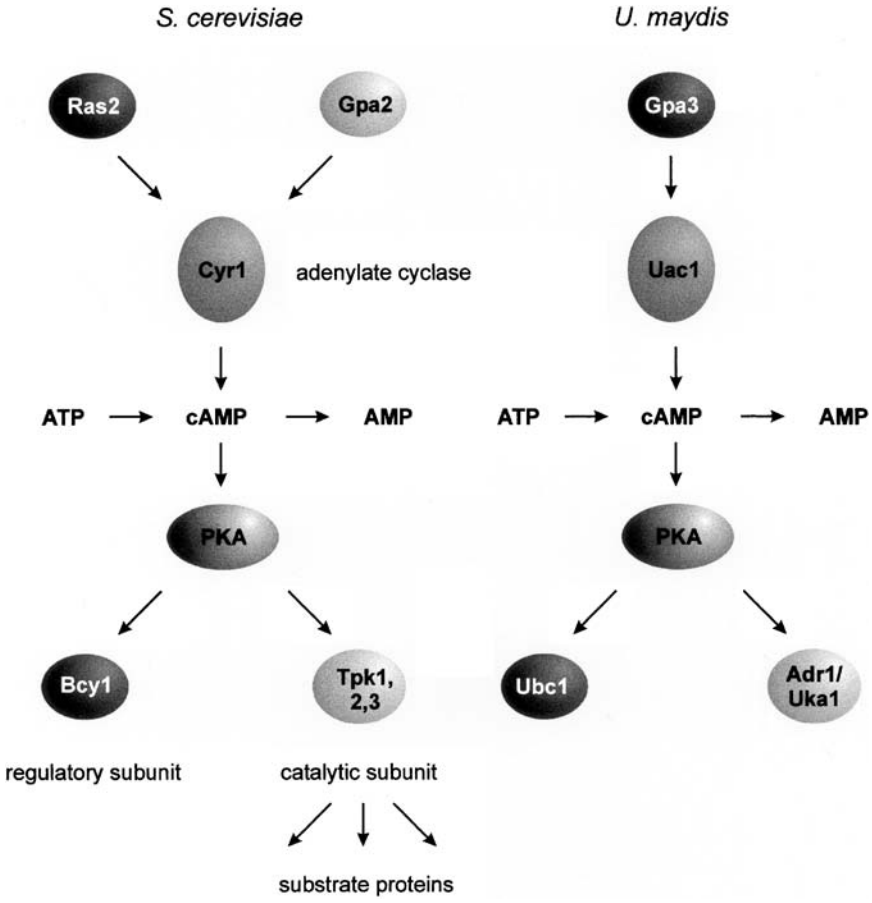

Figure 2 Diagrammatic representation of the cAMP signaling pathway that controls filamentous growth in *S. cerevisiae* (left) and homologues involved in pathogenic development of *U. maydis* (right). In *S. cerevisiae* adenylate cyclase (Cyr1p) can be activated via two pathways involving the small G protein Ras2p or the Gα subunit (Gpa2p) of heterotrimeric G proteins resulting in an increased cAMP level. cAMP binds to the regulatory subunit (Bcy1p) of PKA causing its dissociation from the catalytic subunits (Tpk). The Tpk subunits can then phosphorylate transcription factors and other substrate proteins. The components of the *U. maydis* cAMP pathway include the Gα subunit, the adenylate cyclase Uac1, the regulatory subunit of PKA Ubc1, and the catalytic subunit of PKA Adr1/Uka1. Mutations in this pathway affect cell morphology and pathogenic development.

(Yang and Dickman 1999), suggesting that signaling via cAMP may be a general aspect of the virulence of fungal pathogens towards their hosts. This is also supported by the finding that in *Trichoderma harzianum*, a biocontrol agent that has been used against a number of plant pathogenic fungi, cAMP increases coiling, one of the typical changes accompanying mycoparasitism (Omero et al. 1999). In nonpathogenic fungi like *N. crassa* a PKA pathway regulates growth polarity (Dickman and Yarden 1999), in *A. niger* the catalytic subunit of PKA regulates colony morphology and sporulation (Bencina et al. 1997) and it was demonstrated that *pkaA* (encoding the PKA catalytic subunit) is involved in conidiation and secondary metabolism, e.g., sterigmatocystin production in *A. nidulans* (Shimizu and Keller 2001).

5 PATHWAY CROSSTALK

Analysis of signaling in fungi has shown that interactions frequently exist between cAMP signaling and MAPK pathways involved in virulence, mating, morphogenesis, and stress response (Kronstad et al. 1998). In the yeast *S. cerevisiae*, signaling through the G protein Ras2p is thought to activate both the cAMP as well as the Kss1p MAPK pathways to influence filamentous growth (Kronstad et al. 1998). As already mentioned previously, *M. grisea* MAPK (Pmk1), involved in appressorium formation, has been identified and it was shown that cAMP is able to restore early stages of appressorium formation in *pmk*-negative mutants (Xu and Hamer 1996). In *U. maydis*, both the Ubc3 (Kpp2) MAPK as well as cAMP signaling regulate mating,

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filamentation, and therefore, also pathogenicity as in *M. grisea* (Mayorga and Gold 1999; Müller et al. 1999).

6 FUNGAL MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS

Mitogen-activated protein (MAP) kinases belonging to the serine/threonine protein kinases are involved in transducing a variety of extracellular signals and regulating growth and differentiation processes (Dickman and Yarden 1999; Xu 2000).

MAPK cascades are evolutionarily conserved in all eukaryotes from yeast to human. They are typically organized in a three kinase architecture consisting of a MAPK, a MAPK activator (MEK, MKK, or MAPK kinase), and a MEK activator (MEK kinase = MEKK or MAPK kinase kinase) (Figure 3). Transmission of the signal occurs by sequential activation of these kinases by phosphorylation (Banuett 1998; Schaeffer and Weber 1999).

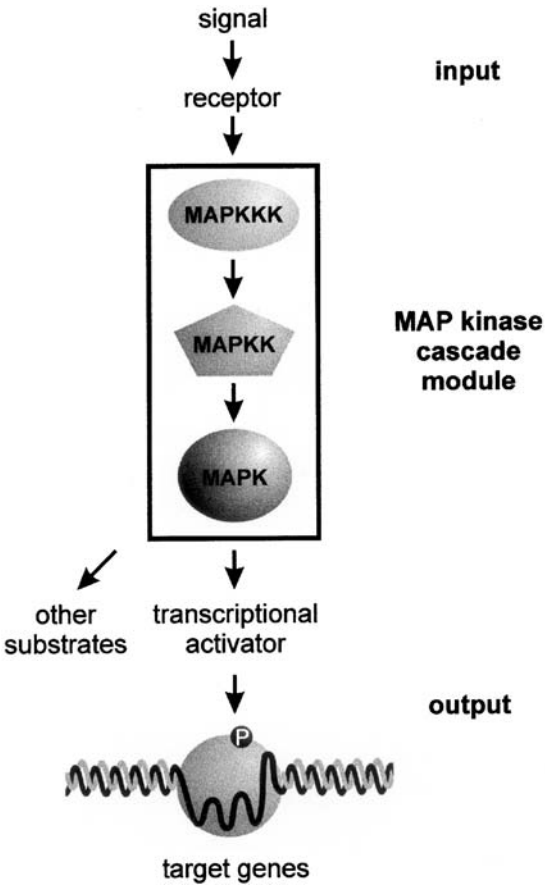

Figure 3 The MAPK module lying in the heart of many signaling pathways in eukaryotes. The MAPK cascade consists of MAPKKK, MAPKK, and MAPK and is stimulated by receptor-mediated extracellular signals. The activated MAPK phosphorylates substrates including transcriptional activators which results in altered gene expression and protein activity.

In *S. cerevisiae*, six independently acting MAP kinases are known and these share extensive sequence identity among themselves and with respective mammalian MAPKs. Five of these have already been associated with the specific responses of regulating mating (MAPK = Fus3p), filamentation (Kss1p), response to osmotic stress (Hog1p), cell wall remodeling (Slt2p), and sporulation (Smk1p). Some MAPKKK–MAPKK–MAPK cascades share several components, and, for example, Ste11p (=MAPKKK) is involved in three MAPK modules responding to pheromones (Ste11p–Ste7p–Fus3p), high osmolarity (Ste11p–Pbs2p–Hog1p), and in the invasive growth response (Ste11p–Ste7p–Kss1p) (Madhani and Fink 1998). Specificity is achieved by activation of different MAPKKK–MAPKK–MAPK modules in response to different stimuli, through the formation of multiprotein complexes (by e.g., using scaffold proteins like Ste5p) that are specific to each pathway or by using anchor proteins that localize their binding partners to specific subcellular compartments or substrates (Atienza et al. 2000; Garrington and Johnson 1999; Madhani and Fink 1998; Whitmarsh and Davis 1998).

There are relatively few examples of characterized MAP kinase modules in filamentous fungi. Recently, Han and Prade (2002) reported the reconstruction of the whole *A. nidulans* salt stress-controlling MAP kinase pathway, based on homology analysis with known yeast genes involved in the HOG pathway.

The importance of MAP kinase cascades for mating and virulence has been described in several plant pathogenic fungi and selected examples are given here. MAP kinases belonging to the yeast extracellular signal-regulated kinase (YERK1) subfamily (Kültz 1998) have been shown to play key roles in the formation of infection structures and in invasive growth of phytopathogenic fungi including the cereal leaf pathogen *M. grisea* (Xu and Hamer 1996) and the maize pathogen *U. maydis* (Kahmann et al. 1999; Mayorga and Gold 1999; Müller et al. 1999), in which the infection process is connected to development of the telemorph.

The MAPK pathways from *U. maydis* are quite well characterized and several components of MAPK modules have been identified (Figure 4). Ubc4, a MAPKKK homolog, and Ubc3, a MAPK homolog, were identified by screening for suppressors of the *uac1* adenylyl cyclase mutation (Mayorga and Gold 1999). *Ubc3* was isolated by a degenerate PCR approach and termed *kpp2* (Müller et al. 1999). Ubc3/Kpp2 was shown to be related to Pmk1 of *M. grisea* and Fus3p of the *S. cerevisiae* pheromone response pathway. In addition to filamentous growth the *ubc3/kpp2* gene is required for pheromone response and for full virulence in *U. maydis* (Mayorga and Gold 1999) and is involved in transmitting the pheromone signal to the transcription factor Prf1 (Müller et al. 1999). However Δ*ubc3/kpp2* mutant cells still respond to exogenous cAMP and it was suggested that the Kpp2/Ubc3

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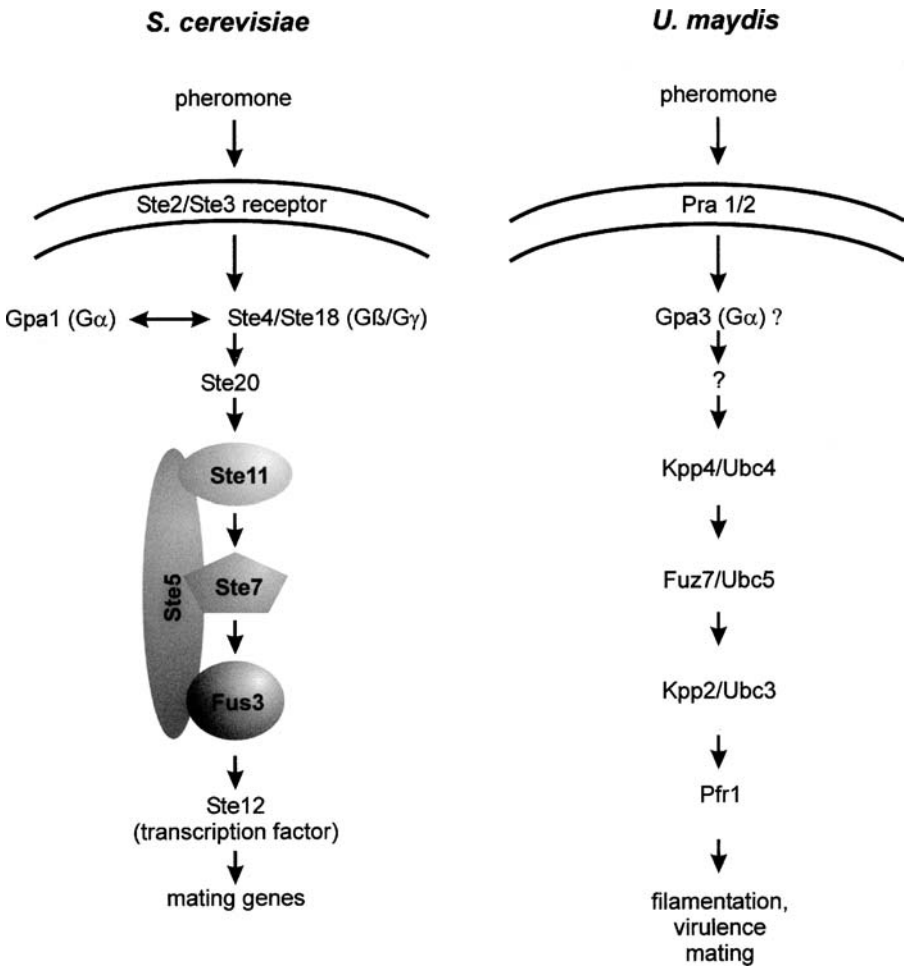

Figure 4 Simplified diagram of the mating MAPK pathway of *S. cerevisiae* (left) and homologues involved in mating and pathogenic development of *U. maydis* (right).The yeast pheromone response pathway is activated upon binding of pheromone to the GPCRs Ste2p or Ste3p, respectively. The scaffold protein Ste5p holds together the MAPK cascade components Ste1p, Ste7p, and Fus3p. MAPK activity results in phosphorylation of Ste12p and subsequent activation of the pheromone response genes. In *U. maydis*, upon pheromone binding to the GPCR (Pra1 or Pra2) the signal is transmitted to a MAPK module containing the MAP kinase Kpp2 that leads to activation of the transcription factor Prf1 resulting in mating, filamentation, and subsequent virulence.

MAP kinase may have multiple targets (including Pfr1) for regulating pathogenic development (Xu 2000). Furthermore, as Δ*ubc3/kpp2* mutants are not completely sterile and still cause lesions, it was suggested that MAP kinases belonging to the same or different pathways may exist and act together with *kpp2* in *U. maydis* to regulate mating and pathogenic development (Kahmann et al. 1999; Müller et al. 1999). Recently, Kahmann and coworkers (2002, 6th European Conference on Fungal Genetics, Pisa, Italy) reported on the cloning of *kpp6* encoding a second MAP kinase that was involved in the pathogenicity but not the mating of *U. maydis*. In addition, Fuz7, a MAPKK homolog of *S. cerevisiae* Ste7p, has been isolated from *U. maydis* and been shown to be necessary for mating- and virulence-related processes including conjugation tube formation, filament formation, maintenance of filamentous growth, *in planta* tumor formation and teliospore formation (Banuett and Herskowitz 1994; Lengeler et al. 2000). At this time it is not completely clear if Fuz7 and Ubc3 are components of the same MAP kinase cascade (Xu 2000).

In *M. grisea* two MAP kinases (Pmk1 and Mps1) play pivotal roles in fungal pathogenicity: Pmk1 (related to *S. cerevisiae* Fus3p and *U. maydis* Kpp2/Ubc3) is essential for appressorium formation (with interconnections to the cAMP pathway, see previously) and invasive growth in plants, but has no effect on development and mating and Mps1 (related to *S. cerevisiae* Slt2p) is required for plant penetration and has a role in regulating cell wall remodeling and polarity

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establishment. Δ*mps1* mutants fail to penetrate plant cuticles, although they still generate turgor pressure and activate plant defense responses (Xu and Hamer 1996; Xu et al. 1998). A third MAPK-encoding gene (*osm1*) was recently identified in *M. grisea* and is functionally related to the *S. cerevisiae HOG1* gene. Δ*osm1* mutants of *M. grisea* showed unaltered glycerol accumulation and turgor generation in appressoria and were fully pathogenic (Dixon et al. 1999).

Takano et al. (2000) used a PCR-based screen with degenerate oligonucleotide primers to isolate a MAP kinase (Cmk1) from *Colletotrichum lagenarium*. This kinase is responsible for appressorium formation and pathogenicity on cucumber leaves and could complement infection-structure formation of a *pmk1* mutant of *M. grisea*. This led to the assumption that a general pathway exists for signaling the formation of fungal infection structures. The MAP kinase Chk1 was isolated from *Cochliobolus heterostrophus*, a corn pathogen which also develops appressoria by applying the same approach. As Δ*chk1* mutants show a pleiotropic phenotype it was suggested that this MAPK may be involved in several developmental processes (Lev et al. 1999). The *bmp1* gene encoding a MAP kinase required for pathogenesis was isolated from the gray mold fungus *B. cinerea* and shown to be very similar to the *M. grisea* Pmk1 (Zheng et al. 2000). MAP kinases have been identified and characterized from *Fusarium* spp. a soilborne fungal pathogen attacking the host plant through its roots. Li et al. (1997) cloned a mitogen-activated protein kinase (FsMAPK) from *F. solani* f.sp. *pisi* T8, and Di Pietro et al. (2001) isolated *fmk1* encoding a MAPK essential for pathogenicity of *F. oxysporum*, and belonging to the same subfamily as the *S. cerevisiae* Fus3p and *M. grisea* Pmk1. Two MAPK genes, *mpk1* and *mpk2*, have also been isolated from the barley powdery mildew fungus *Blumeria graminis* (Zhang and Gurr 2001). Nearest-neighbor-joining phylogenetic analyses of the respective proteins showed that Mpk1 is most closely related to the group of MAP kinases comprising *B. cinerea* Bmp1 (Zheng et al. 2000), *C. lagenarium* Cmk1 (Takano et al. 2000), *F. oxysporum* Fmk1 (Di Pietro et al. 2001), *F. solani* FsMAPK (Li et al. 1997), and *M. grisea* Pmk1 (Xu and Hamer 1996). *B. graminis* Mpk2 lies closest to *M. grisea* Mps1 (Xu et al. 1998) and *A. nidulans* MpkA, which is involved in polarized growth of the fungus (Bussink and Osmani 1999).

Summarizing these data the regulation of virulence including infection structure formation, such as appressoria, and the production of cell-wall degrading enzymes by MAPK signaling pathways using Pmk1-like MAP kinases seems to be widely conserved in plant pathogenic fungi.

7 CALCIUM/CALMODULIN-DEPENDENT SIGNALING IN FUNGI

Calmodulin is a highly conserved calcium binding protein found in all eukaryotic cells, and it is involved in many Ca^{2+} dependent processes including signal transduction, motility, secretion, and cell cycle regulation (James et al. 1995). Calmodulin has also been identified in a number of fungi where it was found to be associated with functions such as cell proliferation and differentiation in several cases (Warwar et al. 2000). Mach et al. (1998) demonstrated that in the industrially applied filamentous fungus *Trichoderma reesei* Ca^{2+}/calmodulin is required both for the transcription as well as the secretion of xylanases and in *A. nidulans* it was shown that deletion or delayed expression of the Ca^{2+}/calmodulin-dependent kinases slows entry into the cell cycle but does not completely block spore germination (Joseph and Means 2000).

The role of calmodulin in plant pathogenic fungal development and during plant infection is not well established. In *M. grisea*, appressorium formation but not germination is completely blocked in the presence of calcium chelators, ionophores, and calcium regulators, suggesting that a calcium/calmodulin-dependent signaling system is involved in appressorium formation (Lee and Lee 1998). In *Colletotrichum gloeosporioides*, calmodulin expression was shown to be triggered by surface contact, resulting in appressorium formation (Kim et al. 1998) and for *C. trifolii* it is reported that the calmodulin gene is most highly expressed during conidial germination and appressorium development and that antisense expression of the *cam* gene impairs pre-penetration development (Warwar et al. 2000).

For *C. parasitica* regulatory pathways including the IP_3^- Ca^{2+}–calmodulin–calcineurin pathway and the general amino acid control pathway that is activated in response to amino acid starvation were also found to be important in regulating pigment production and virulence (Lengeler et al. 2000; Wang et al. 1998).

8 CONCLUSIONS

Conserved signaling pathways seem to have been developed from fungal pathogens for regulating pathogenicity-related functions. Besides the previously described examples of plant pathogens, some evidence for similar signaling mechanisms in human and animal pathogenic fungi are given. In the basidiomycetous yeast *C. neoformans*, a Gα protein transmitting the cAMP signal has been found to be required for virulence (Alspaugh et al. 1998). The pathogenic yeast *Candida albicans* undergoes a yeast-to-mycelium transition in response to various environmental signals, including serum and the presence of macrophages in which cAMP has been implicated to be involved (Niimi 1996). Furthermore, the *CST20* gene of *C. albicans* has been isolated and shown to complement a deletion of the *STE20* protein kinase gene in *S. cerevisiae*. Cells deleted for *CST20* were less virulent in a mouse model, suggesting that more than one signaling pathway can trigger the hyphal development necessary for virulence in *C. albicans* (Leberer et al. 1996).

Interestingly, these observations closely parallel the regulation of fungal virulence in plant pathogens such as *C. parasitica*, *U. maydis*, *M. grisea*, and *C. trifolii*, where

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appressorium development or plant tissue colonization include MAPK elements of the pheromone response pathway of *S. cerevisiae*, as well as components of the Ras-adenylate cyclase pathway and heterotrimeric G proteins. The fact that such a diverse group of fungi employs similar signaling elements to regulate virulence argues for a conservation of function of both the cAMP as well as MAPK signaling pathways involved.

ACKNOWLEDGEMENTS

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16

Application of Genetic Engineering for Strain Improvement in Filamentous Fungi

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1 INTRODUCTION

Inherent characteristics of filamentous fungi such as the ability to grow on cheap cultivation media and excellent protein secretion makes them competitive production organisms when compared to the eukaryotic yeast, animal, plant, and insect cell systems. One of the principal aims in fungal strain improvement discussed in this Chapter is yield enhancement of a particular protein that is secreted into the cultivation medium. Early strain development programs were based on the application of random mutagenesis and screening of the mutagenized cells for specific characteristics (Rowlands 1984). This method has been particularly successful in the isolation of high protein-secreting mutant strains that are currently being used as expression hosts for both homologous and heterologous gene products (Dunn-Coleman et al. 1991; Nevalainen et al. 1994a; Mäntylä et al. 1998). Currently, molecular techniques such as increasing the gene copy numbers as well as performing gene knock-outs are being applied for the further refinement of fungal products and production systems [e.g., Mäntylä et al. (1998)]. A number of research programs discussed in this Chapter and elsewhere in this volume, have been initiated in order to better understand the complex genetic networks involved in gene expression and protein secretion in filamentous fungi.

The list of mainstream fungi currently used for product manufacture featuring *Aspergillus* (enzymes, organic acids, and fermented foods), *Trichoderma* (enzymes, biological control), *Mucor* (enzymes), *Rhizopus* (enzymes), *Penicillium* (antibiotics, cheese), and *Cephalosporium* (antibiotics) has remained unchanged for quite some time. Expression systems based on, for example, *Chrysosporium lucknowense* (van Zeijl et al. 2001) and *Fusarium venenatum* (Blinkovsky et al. 1999) have been more recently developed.

Enhanced manufacture of gene products from genetically tailored strains of industrial fungi such as *Trichoderma reesei* and *A. niger* is a routine procedure nowadays. Further improvement of protein production draws from basic research into gene regulation, protein glycosylation, hyphal morphogenesis and polarized growth, function of the fungal secretory pathway at both genetic and ultrastructural level, fermentation physiology, and studies into functional genomics. Considerable amount of research is directed to revealing bottlenecks in the production of heterologous gene products of which the yields have typically remained 10–1000 times lower than those for homologous proteins [reviewed in Archer and Peberdy (1997), Conesa et al. (2001), Keränen and Penttilä (1995), and Radzio and Kück (1997)].

2 POINTS TO CONSIDER IN STRAIN IMPROVEMENT

Recombinant DNA technology provides the tools for driving gene expression from strong promoters, increasing the gene dosage, deletion of unwanted genes from the fungal genome, manipulation of metabolic pathways, introduction of novel properties, and improving the performance of proteins themselves.

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2.1 Transformation Strategies

Genetic transformation systems have been developed for both ascomycetous and basidiomycetous fungi including gilled basidiomycetes (Finkelstein 1992; Irie et al. 2001 and references therein), therefore, making possible the genetic modification of industrial protein producers as well as fungi targeted for food and biological control applications. A "universal" transformation method successfully applied for a large number of filamentous fungi is polyethylene glycol mediated DNA uptake by protoplasts. Other methods used previously include electroporation of protoplasts (Goldman et al. 1990; Ward et al. 1989) and incubation of germinating conidia in a lithium salt (Dhawale et al. 1984). More recently, microprojectile bombardment of intact conidia with gold or tungsten particles coated with DNA has been introduced to filamentous fungi [e.g., Armaleo et al. (1990), Hazell et al. (2000), Herzog et al. (1996), and Lorito et al. (1993)]. The biolistic method, especially when performed on uninucleate haploid conidia, has proven successful in bypassing the time consuming repetitive purification of transformed protoplasts and providing a high percentage of stable transformants. Recently, a relatively high frequency transformation of both fungal conidia and protoplasts has been reported using *Agrobacterium* T-DNA (de Groot et al. 1998).

In general, the transformation frequencies with filamentous fungi, notwithstanding the method used, are typically in between 10 and 100 transformants per microgram of DNA. These are considerably lower to the comparative transformant numbers, for example, in yeast that can reach $10^5\ \mu g^{-1}$ of transforming DNA or the prokaryote *Escherichia coli*. Therefore, current molecular evolution programs, e.g., for enzymes of fungal origin are mainly carried out in *E. coli* where transformation frequencies and robotized screening methods are advanced enough to allow for compilation of mutant libraries and effective high throughput screening of gene products.

Dominant selection markers such as the *Aspergillus nidulans amdS* gene (Kelly and Hynes 1985) encoding the acetamidase enzyme and bacterial antibiotic resistance markers, such as the *hph* gene encoding resistance to hygromycin B (Punt et al. 1987) have proven to be functional over a wide range of fungi. The gene of interest may be included in the same vector as the transformation marker or introduced in a fungal strain by cotransformation. Retaining a possibility for several consecutive transformations of the same strain for further manipulation adds to the flexibility of a given system. In an industrial setting, aiming for high product yields, high secreting strains are being used as transformation hosts. Transformation of filamentous fungi is discussed in detail in Chapter 9 by RL Mach.

2.2 Promoters and Secretion Signals

A strong promoter capable of driving efficient transcription of DNA under required growth conditions is a key feature of an expression vector. The lack of a suitable promoter system may restrict attempts to develop new expression systems and hosts for protein production. Most of the published studies concerning high level gene expression have been carried out with species of *Aspergillus* and *Trichoderma* [reviewed in van den Hondel et al. (1985) and Penttilä (1998)]. Gene promoters used for recombinant expression can be roughly divided in two categories, inducible and constitutive. Among the strongest inducible promoters, regulated by carbon catabolite repression are the glucoamylase A promoter (*glaA*) of *A. niger* var. *awamori* (Ward et al. 1990) and the *T. reesei* cellobiohydrolase 1 (*cbh1*) promoter (Harkki et al. 1991). A constitutive promoter used across fungal species is the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase *gpdA* (Punt et al. 1991). So far, natural constitutive or carbon catabolite repression insensitive promoters comparable in strength to the *cbh1* and *glaA* have not been described.

Among recently discovered promoters are *Penicillium chrysogenum phoA* encoding a secreted phosphate-repressible acid phosphatase (Graessle et al. 1997) and a trypsin-like protease promoter from *Fusarium oxysporum* (Wiebe et al. 1999). The work by Gordon et al. (2001) describing combined use of a growth rate depending promoter (*A. niger glaA*) and a growth rate independent promoter (trypsin-like protease) in batch and fed-batch bioreactor cultures of *F. oxysporum* producing a recombinant glucoamylase shows an important line of research that effectively combines molecular biology, gene regulation, and fungal physiology with fermentation technology to improve product yields. When aiming for high yields of secreted products, physiological state of the organism must be in line with the requirement. For example, when growing on a simple and easily metabolized carbon source such as glucose, low amount of protein is found to be secreted into the cultivation medium whereas growth on a polymeric sugar such as cellulose induces high-level production of secreted enzymes for substrate degradation outside the fungal mycelium. Examples relating physiology to product yields are discussed in Section 4.

N-terminal signal peptides introduce proteins into the secretory pathway [see references in Conesa et al. (2001)]. Signal peptides share a common architecture and are generally not believed to have a major impact on the yields of recombinant gene products. In a typical situation, a signal sequence homologous to the production host is used. Similarly, even though promoters especially from Ascomycetous fungi are interchangeable at least to some extent, a promoter endogenous to the production host is used for high yield expression.

An interesting approach into modeling of promoter activity in *A. nidulans* has been described by Agger and Nielsen (1999) who developed a genetically structured model for the expression of the inducible alcohol dehydrogenase I (*alcA*) promoter. The model was successfully shown to simulate the experimental data. In the future, it may be possible to simulate regulation of a given promoter using

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computer programs and thereby carry out some of the tedious laboratory experiments *in officio*.

2.3 Gene Regulation and Transcription Factors

Most of the strong promoters used for protein production in filamentous fungi are controlled fairly strongly by transcriptional regulation. This reflects the fact that the production promoters are often derived from genes encoding extracellular hydrolytic enzymes such as cellulases, xylanases, and amylolytic enzymes, and constitutive production of these enzymes would be a waste of energy for the fungus. Thus, in the presence of an easily utilizable carbon source such as glucose, expression is repressed and induction is needed for high-level production. For instance, production using cellulase promoters of *Trichoderma* can be obtained on media containing cellulose, plant material, or lactose (Kubicek and Penttilä 1998). The difference in expression levels between the repressed and induced conditions can be several 1000-fold as shown for the cellulase promoter *cbh1* of *T. reesei* (Ilmén et al. 1997).

Strict regulation, in particular glucose repression (carbon catabolite repression) is not necessarily beneficial under production conditions. In fact, many of the production strains obtained by classical mutagenesis are to some extent glucose-derepressed and have been particularly selected or screened for these characteristics (Dunn-Coleman et al. 1991; Montenecourt et al. 1981). Mutant strains enable production strategies where the cheap and defined substrate glucose is used as a carbon source or a cosubstrate that is fed at low levels to the fermenter without a repressing effect. Furthermore, glucose-derepressed strains may also produce higher levels of a particular enzyme in inducing conditions than the nonmodified strain.

Modulation of glucose repression either at the strain or promoter level is, therefore, of importance in fungal strain development. Glucose repression in filamentous fungi is mediated by the repressor protein CRE that binds to the sequence 5′-SYGGRG in the target promoters via the DNA binding domain consisting of zinc fingers (Cubero and Scazzocchio 1994). The *cre* gene has been characterized from a number of fungi including *Aspergillus* (Dowzer and Kelly 1991; Drysdale et al. 1993), *Trichoderma* species (Ilmén et al. 1996b; Strauss et al. 1995; Takashima et al. 1996), and *Humicola grisea* (Takashima et al. 1998). Interestingly, the hypercellulolytic mutant strain RUT-C30 of *T. reesei* (Montenecourt and Eveleigh 1981) was later found to be mutated in the *cre1* gene (Ilmén et al. 1996b). Mutations in *cre* lead to expression on glucose to varying extent of the otherwise glucose-repressed genes such as the *A. nidulans alcA* encoding alcohol dehydrogenease (Felenbok et al. 2001) and cellulase and various hemicellulase genes of *T. reesei* (Ilmén et al. 1996b; Margolles-Clark et al. 1997) and *Aspergillus* species (Ruijter et al. 1997). In addition, xylose repression is mediated by CRE shown for hemicellulases in *A. niger* (de Vries et al. 1999). Similarly, if the CRE protein binding sites in a promoter are mutated, expression of that particular gene becomes derepressed on glucose (Ilmén et al. 1996a; Mach et al. 1996). Since, many unwanted proteins are secreted on cheap industrial carbon sources that provoke high level enzyme production, production of a single enzyme component on for instance glucose would be desired. However, none of the modified promoters currently described would give high enough expression levels on glucose for industrial purposes.

There are several other regulatory proteins described in fungi whose overexpression (or mutation or deletion) could provide means towards improved protein production. The first described was the activator of the *A. nidulans alcA* gene, *alcR* (Felenbok et al. 2001). This gene regulates genes involved in ethanol utilization. Overexpression of the regulator *alcR* improved expression of the *alcA* gene demonstrating that the positively acting regulatory factors may become limiting for expression, which could be particularly true for strains bearing multiple copies of the expression cassette (Mathieu and Felenbok 1994). AMYR (Petersen et al. 1999) and SREB (Tani et al. 2000) are positive factors involved in induction of amylase genes. Another important regulatory protein is XlnR, which is a powerful activator of several cellulase and hemicellulase genes of *A. niger* and *A. oryzae* and most likely also has homologs in other filamentous fungi (Marvi et al. 2002; van Peij et al. 1998b). Overexpression of this gene in *A. niger* increases expression of xylanases and cellulases (Gielkens et al. 1999a,b; van Peij et al. 1998a). Furthermore, two new regulatory proteins affecting fungal enzyme production have been discovered. These are the *T. reesei* cellulase and xylanase regulator *ace1*, whose deletion increases expression of the genes in the presence of cellulose or the inducer sophorose (Aro et al. 2002; Saloheimo et al. 2000), and *ace2* which is involved in activation of the genes on cellulose (Aro et al. 2001). For all these factors (putative) binding sites in the target promoters have been determined at least by *in vitro* binding assays.

Yet another important regulatory factor of fungi is encoded by the *A. nidulans pacC* gene which acts as an activator for alkaline-expressed genes at alkaline pH and prevents expression of genes that are normally expressed in acidic conditions (Caddick et al. 1986; Tilburn et al. 1995). Proteases, xylanases (MacCabe et al. 1998), and arabinofuranosidase (Gielkens et al. 1999a) have been reported to be controlled by *pacC*. Additional potentially useful factors are encoded by the *areA* genes of *Aspergilli* [see Christensen et al. (1998)] and the homologs that are involved in utilization of nitrogen sources.

2.4 Effect of Steady-State MRNA Levels on Product Yield

Good steady-state levels of a particular messenger RNA are of primary importance in obtaining good product yields. These levels are affected by transcription efficiency and mRNA

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stability. However, in case of heterologous gene products, high amounts of mRNA may not guarantee respective high protein yields indicating that additional factors are involved [e.g., Gouka et al. (1997) and Saloheimo and Niku-Paavola (1991)]. In a number of cases, fusion of a homologous carrier such as *glaA* or *cbh1* to the 5′ end of the heterologous gene has resulted in a considerable increase in product yields (Gouka et al. 1997; Nyyssönen and Keränen 1995). This may originate from an increase in the mRNA stability or effective localization of the hybrid mRNA resulting from the gene fusion. Reports on the mRNA localization have started to emerge for filamentous fungi only recently (Nykänen et al. 1997; 2002a,b) suggesting that localization of mRNA may have an effect on translational activity and thereby product yields.

2.5 Effects of Codon Usage

The use of synonymous codons can vary widely between different genes and organisms. Therefore, when aiming at efficient expression of proteins of interest in a heterologous host, analysis of the codon usage pattern, and appropriate modification of codons according to the codon preference of the host is highly recommended. Thus far, the effects of codon usage on heterologous gene expression in filamentous fungi have not been studied in detail and currently there are only a few examples where the codon usage in the incoming gene has been optimized (Moralejo et al. 1999; Te'o et al. 2000). In a study of Te'o et al. (2000), successful expression in *T. reesei* of an AT rich xylanase gene from the thermophilic bacterium *Dictyoglomus thermophilum* was achieved after changes were made to 115 nucleotides in the 630 bp *xynB* coding region. The overall AT content of the *D. thermophilum xynB* is 61% compared to less than 40% in a typical *T. reesei* cellulase gene. In addition, *xynB* prefers A or T at the third codon position (Morris et al. 1998).

The above example highlights some difficulties in the expression of genes with high AT content in a fungal host. Pre-mRNA processing in eukaryotes involves polyadenylation of the message following recognition of the AU-rich polyadenylation signal. In the expression of AT-rich heterologous genes, AU-rich elements may be interpreted as polyadenylation signals by the fungal host, which in turn would result in formation of truncated mRNA (Gouka et al. 1997; Romanos et al. 1992). In eukaryotes, mRNA stability and translability are linked to the level of mRNA polyadenylation (Wickens et al. 1999). In protein translation, the correct codon usage may facilitate translation initiation and efficiency. Codon modification circumvents a potential problem of the availability of isoacceptor tRNAs in the host suitable for codons of the foreign gene.

2.6 Translational Fusions

A strategy based on translational fusion of a (heterologous) protein to an endogenous effectively secreted carrier protein has been applied widely in order to improve the yields of heterologous gene products. The N-terminal fungal fusion partner has been proposed to stabilize the recombinant mRNA, facilitate the translocation of foreign proteins in the secretory pathway, and protect the heterologous part from degradation (Gouka et al. 1997; Nyyssönen et al. 1995; Penttilä 1998).

The carriers can be either intact proteins (Roberts et al. 1992) or more frequently, a domain of an endogenous protein is replaced by the heterologous protein. Examples are provided by the work of Ward et al. (1990) where calf chymosin was produced in *A. niger* as a fusion to glucoamylase (GLA) and by Jeenes et al. (1993) where the substrate binding domain of GLA was replaced by sequences encoding hen egg-white lysozyme. Similar approaches have been described for *T. reesei*, in which parts of the cellobiohydrolase I (CBHI) where fused to calf chymosin (Harkki et al. 1989) or in which the cellulose binding domain of CBHI was replaced by sequences encoding antibody Fab fragments (Nyyssönen et al. 1993). In general, translational fusions have been beneficial for the improvement of synthesis of gene products originating from nonfungal organisms by 5–1000 fold up to hundreds of milligrams per liter, but appear not necessary for efficient expression of fungal proteins in heterologous fungal hosts (Conesa et al. 2000; Faria et al. 2002) where gram(s) per liter levels have been obtained. Specific sites for product cleavage such as factor X or Kex2p can be added for the separation of the heterologous part from the carrier protein (see Section 3.5). In addition, short purification tags such as 6xHIS may be included in the expression constructs. In a study by Nykänen et al. (2002b,c), secretion of calf chymosin through the cell wall of *T. reesei* was greatly facilitated by the endogenous CBHI fusion partner.

The N-terminal glycosylation, which is provided by the most frequently used endogenous fusion partners such as GLA and CBHI has been considered favorable for protein solubility and proper folding (Sagt et al. 2000). However, there are also examples where the gene-fusion strategy has been unsuccessful in secretion of heterologous gene products from filamentous fungi, probably because of misfolding and/or incorrect processing in the secretory pathway. One such example is the oligomeric hTNF-alpha (Krasevec et al. 2000).

2.7 Gene Targeting, Multiple Copies, and Knock-Outs

Targeted integration of the expression construct in an endogenous locus known to accommodate a gene that is effectively transcribed has, in some cases, resulted in increased expression of a gene product of interest (Harkki et al. 1991). For example, targeting several copies of the *egl1* gene into the *cbh1* locus has been utilized to modify cellulase profiles in the industrial production strains of *T. reesei*

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(Karhunen et al. 1993; Mäntylä et al. 1998). However, there are also examples where the integration site has had no notable effect on product yields (Nevalainen et al. 1994b). Therefore, no universal rule can be provided. In addition to directly increasing product yields, targeted integration can be used to delete unwanted genes. For example, *Aspergillus* strains deficient in aspergillopepsin have been constructed to be used as hosts for heterologous gene products (Berka et al. 1990a; Moralejo et al. 1999, see discussion in Section 3.8).

Integration efficiency has been shown to relate to the length of the DNA flanking the 5′ and 3′ ends of the gene to be integrated (van den Hondel et al. 1991; Suominen et al. 1993). In the vector construction, a provision for targeted integration should be retained to add to the flexibility of the use of the expression system. Recently, PCR based techniques have been introduced for efficient gene replacement in *A. nidulans* (Chaveroche et al. 2000) and the plant pathogen *Ashbya gossypii* (Wendland et al. 2000). In case of *A. gossypii*, short sequences with 40–46 bp homology to two sequences of the targeted gene (*RHO4*) provided sufficient homology for homologous recombination. The strategy described for *A. nidulans* uses first *in vivo* recombination in *E. coli* of a recombinant cosmid carrying the fungal gene of interest and a PCR-generated transformation marker flanked by short, 50 bp regions of homology to the target DNA. According to Chaveroche et al. (2000), the method results in 50% efficiency in creating knock-out mutants and is also effective when using circular cosmid DNA. The applicability of the described techniques to industrially relevant fungi remains to be studied. If functional, the strategies discussed previously will provide a rapid method to carry out promoter replacements or gene fusions of interest.

Fungal transposons have been used for large scale gene disruption. For example, the transposon-arrayed gene knock-outs (TAGKO) have been applied for the analysis of pathogenicity-related genes in the plant pathogenic fungus *Magnaporthe grisea* (Hamer et al. 2001a,b).

The number of gene copies introduced in a fungal genome is relative to increased synthesis of the corresponding gene product at least to a certain point (see discussion on gene regulation in Section 2.3). In *T. reesei*, endoglucanase activity of transformants did not increase further after inserting three copies of the *egl1* gene in the resident *cbh1* locus (Karhunen et al. 1993). Multicopy expression from the cellulase promoter *cbh1* of a heterologous chitinase in *T. reesei* did not lead to increased production either and indications were obtained that the regulatory factors may have become limiting, since expression of the endogenous *cbh1* gene was reduced as compared to the host itself (Margolles-Clark et al. 1996). In *Aspergillus*, higher copy numbers have been reported to be effective in terms of improving gene product yields (Moralejo et al. 1999).

In addition to aiming at achieving good transformation frequencies, stable integration of the transforming DNA in the fungal genome and efficient transcription of target genes, events related to posttranslational processing of proteins are of primary importance for developing effective strain improvement strategies.

3 POSTTRANSLATIONAL EVENTS

The fungal secretory route has been called a "highly productive black box" (Peberdy et al. 1994). A number of current studies are addressing intracellular localization and concurrent processing of homologous and heterologous proteins in the fungal secretory pathway in order to characterize the mechanism and identify bottlenecks for heterologous protein production.

3.1 Secretory Pathway at a Glance

The fungal secretory pathway is depicted in Figure 1. Secretory proteins featuring signal sequences are first entered in the ER where they fold and undergo modifications such as core glycosylation, disulfide bridge formation, and proteolytic processing. From the ER, proteins proceed in secretory vesicles to the Golgi where further modifications may take place. The final stages of secretion involve fusion of the protein-containing vesicles to the plasma membrane and their externalization through the fungal cell wall. Depending on the address tag in the protein-encoding sequence, some proteins may settle in the ER or go to cytoplasmic organelles such as mitochondria and specific vesicles. The fungal secretory pathway is reviewed in more detail in Conesa et al. (2001) and Gouka et al. (1997).

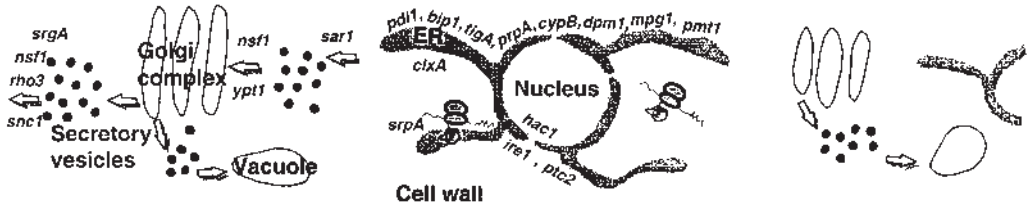

Figure 1 The secretion pathway in filamentous fungi Function of some genes shown are discussed in the text.

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3.2 Chaperones and Foldases in Strain Improvement

Experimental evidence gained so far suggests that several foreign proteins are lost in the secretory pathway because of incorrect processing or misfolding, resulting in their elimination by cellular quality control mechanisms [reviewed in Archer and Peberdy (1997) and Gouka et al. (1997)]. These observations have led to research programs studying cellular pathways for unfolded protein response [UPR; reviewed in Chapman et al. (1998) and Welihinda et al. (1999)] and cloning of genes encoding gene products assisting in protein folding and quality control.

One of the best studied chaperones for which a corresponding gene has been cloned from fungi is the binding protein BiP, a member of the heat shock 70 protein family residing in the ER (van Gemeren et al. 1997; Punt et al. 1998). The BiP is overexpressed by, for example, heat shock, glucose starvation and conditions that induce the UPR [see references in Conesa et al. (2001)]. Several studies have also shown that overproduction of homologous and recombinant proteins induces the expression of *bipA* in *A. niger* and *A. awamori* (Punt et al. 1998 and references therein; Conesa et al. 2002; Ngiam et al. 2000). However, overexpression of *bipA* has not, in general, resulted in higher yields of heterologous proteins tested so far.

Other ER luminal chaperones are the lectin-like soluble calreticulin and its membrane bound counterpart calnexin for which the corresponding gene has been identified in a number of filamentous fungi (Conesa et al. 2002; Juvvadi et al. 2001; Nelson et al. 1999; Roe et al. 1999; 2000). Calnexin overexpression has been shown to increase production of the heterologous *Phanerochaete chrysosporium* manganese peroxidase (MnP) in *A. niger* up to five-fold in the absence of heme (Conesa et al. 2002). Interestingly, the positive effect of calnexin overexpression was lost when the transformant was supplemented with heme. Overexpression of *bipA* had a positive effect on the MnP yields.

Most promising results on overexpression of chaperones and foldases in fungal hosts in order to increase heterologous protein production come from studies involving protein disulphide isomerase (PDI) overexpression. The PDI catalyses the formation and rearrangement of disulfide bridges in proteins during folding (Noiva 1999). Genes encoding protein disulfide isomerase PDI (Hjort 1995; Kajino et al. 1994; Lee et al. 1996; Malpricht et al. 1996; Ngiam et al. 1997; Saloheimo et al. 1999) and other PDI-related enzymes (Jeenes et al. 1997; Wang and Ward 2000), peptidy prolyl isomerases (PPIs) (Derkx 2000; Joseph et al. 1999) have been isolated from filamentous fungi including biotechnologically relevant species. Similarly to Bip, PDI expression is induced by secretion stress and expression of foreign proteins in fungi, for example, antibody fragments in *T. reesei* (Saloheimo et al. 1999). In the expression of thaumatin, a plant protein containing eight disulfide bridges, about five-fold increase in thaumatin yield was obtained in an *A. awamori* transformant showing increased PDI expression. At the same time, the production levels of endogenous α-amylase and acid phosphatase were unaffected. The production levels of the heterologous thaumatin reached 150 mg/l in a fermenter cultivation (Moralejo et al. 2001).

In filamentous fungi, the effect of overexpression of chaperones and foldases on the product yield has been inconsistent and seems to be product dependent. It may imply that the portfolio of foldases and chaperones needed are different for different proteins. In addition, overexpression of particular foldases may interfere with the overall balance of the folding machinery in the cell. This situation clearly shows the difficulty in obtaining conclusive results by observing only a certain group of genes in a complex system. Effects of overexpression of various foldases and chaperones are summarized in Conesa et al. (2001).

Recently, the UPR pathway regulator, *hac*, has been cloned from *T. reesei*, *A. nidulans*, and *A. niger* (Saloheimo et al. 2002) and the activation mechanism studied in detail. Overexpression of this regulator increases expression of Bip and PDI and most likely also other factors under HAC control. In *S. cerevisiae* Hac1p controls, in addition to the folding factors, a variety of cellular phenomena related to protein secretion such as proteolysis through the ERAD pathway (see Section 3.3), and genes involved in glycosylation and in the secretion process itself (Travers et al. 2000). Overexpression of the *T. reesei* factor in the yeast *S. cerevisiae* increased extracellular production of *Bacillus* α-amylase and the endogenous protein invertase (Valkonen et al. 2002). In addition, overexpression of the *A. niger hacA* increased production of a heterologous laccase in *A. niger* several fold. On the other hand, the *T. reesei hac1* gene did not have a beneficial effect on expression of the same laccase in *T. reesei* (Valkonen, personal communication 2002). These data show that albeit improved strains can be obtained by upregulation of the complete UPR pathway, the effect cannot be guaranteed; it may depend on the optimal level of the UPR components and obviously the foreign protein in question. In any case, the fact that transformation in fungi occurs through integration of varying gene copies into the genome resulting in transformants with different expression levels provides a means to screen for optimal expression of the complete UPR pathway or a single cellular factor.

3.3 ER-Associated Protein Degradation Pathway (ERAD)

The intracellular quality control, initiated by inefficient translation, ineffective or incorrect protein translocation, incomplete or inaccurate folding, proteolytic processing, or incomplete glycosylation works effectively in clearing the system of defective gene products. The ERAD eliminates misfolded proteins by their degradation in the cytosol. Defective proteins detected in the ER are transferred to cytosol and targeted for degradation in the 26S proteasome,

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a multienzyme complex by ubiquination (Brodsky and McCracken 1999; Sommer and Wold 1997). Currently, limited information is available on the proteasome of filamentous fungi: one gene, *prs12*, a homolog of mouse regulatory subunit 12 of the 26S proteasome has been characterized from *T. reesei* (Goller and Kubicek 1998). The 26S proteasome has recently been isolated from *T. reesei* (Hauge, personal communication 2001).

3.4 Protein Glycosylation

Glycosylation may have an effect on secretion, structure and stability, immunological properties, intracellular processing and activity and proteolytic degradation of proteins (Lis and Sharon 1993). Considering this and the importance of fungal hydrolases as biotechnologically important enzyme products, surprisingly little information is available on the sites, type, and composition of glycosylation on proteins produced in filamentous fungi. Recent research has addressed the form and content of glycans added to secreted fungal proteins [e.g., Chiba et al. (1993), Maras et al. (1997a,b), and Takegawa et al. (1991) and references therein; Harrison et al. (1998; 2002) and Klarskov et al. (1997)]. In general, the fungal N-linked glycan core has shown to be identical to the mammalian N-linked core ($Man_3GlcNac_2$) and some fungi seem to synthesize large amounts of high-mannose type glycans. There are also reports on the occurrence of a single *N*-acetylglucosamine on the main CBHI of *T. reesei* ALKO2877 and QM9414 (Harrison et al. 1998; Klarskov et al. 1997) which suggests either that strains of *T. reesei* N-glycosylate CBHI differently or, maybe more likely, that the fungus secretes glycan trimming enzymes to the culture medium. This in turn, is important when choosing a suitable host strain or cultivation condition for the synthesis of a particular (heterologous) gene product. There is also evidence that different fungi such as *Trichoderma* and *Aspergillus* glycosylate proteins differently (Maras et al. 1997b and references therein; Nevalainen et al. 1994b), therefore, a choice may be made between different fungal species.

Varying results have been published concerning the importance of N-linked glycosylation for the secretion or stability of extracellular enzymes from filamentous fungi (Chen et al. 1994; Eriksen et al. 1998; Neustroev et al. 1993). For example, N-glycosylation of cellulases of *T. reesei* have been shown not to be obligatory for enzyme activity and secretion (Kubicek et al. 1987) but has an effect on their thermostability and resistance to proteolysis (Merivuori et al. 1985a; Wang et al. 1996). On the other hand, complete inhibition of N-glycosylation of a *Rhizomucor miehei* aspartic proteinase has led to intracellular accumulation of the protein (Murakami et al. 1993).

O-linked glycans of glucoamylase from *A. awamori* (Neustroev et al. 1993), *A. niger* (Gunnarsson et al. 1984), and CBHI of *T. reesei* (Harrison et al. 1998) include di- and trisaccharides containing terminal glucose, mannose, and galactose. It has been suggested that O-linked sugars contribute essentially to the stabilization of, e.g., glucoamylase domains in *A. awamori* and facilitate secretion and activity of *T. reesei* cellulases (Kruszewska et al. 1999; Kubicek et al. 1987; Merivuori et al. 1985a,b). Apart from O- and N-linked glycosylation, further structural diversification may occur by covalent attachment of phosphate, sulfate, acetyl, or methyl groups to the sugar. Biological significance of these modifications is yet to be elucidated.

Reports on the effect of glycosylation on the overproduction of homologous proteins and yields of heterologous gene products are still sparse and somewhat contradictory. It was shown by Ward et al. (1989) that engineering of an N-glycosylation site in the recombinant calf chymosin produced in *Aspergillus*, improved secretion of the heterologous gene product by 10-fold. Similar effect was seen with *A. awamori* producing insulin (Mestric et al. 1996). Kruszewska et al. (1999) reported that overexpression of the mannosylphosphodolichol synthase encoding gene of *S. cerevisiae* in *T. reesei* improved secretion of the main CBHI suggesting that glycosylation was a limiting factor for overproduction. However, Wallis et al. (1999a,b) found a similar pattern of glycosylation in both the wild type and glucoamylase overproducing *A. niger* strain. Despite of the inconclusive nature of reports published so far concerning overproduction, it is evident that glycosylation has a role in protein secretion and protein stabilization in filamentous fungi.

In a wider perspective, for the production of heterologous glycoproteins in filamentous fungi, especially those of therapeutic importance, a good knowledge of the mechanism of protein glycosylation and nature of the glycans produced by the host is elementary. Equally important would be the appropriate modification of fungal glycosylation either by chemical or biological means. Towards this end, the enzymatic mechanism for protein glycosylation in fungi has been studied (Maras et al. 1997b; van Petegem et al. 2001) and genes involved isolated (Kruszewska et al. 1998; 2000; Maras et al. 2000) with a view of dialing up correct glycosylation for a particular gene product synthesized in a filamentous fungal system. Most importantly, fungi appear to produce core glycans suitable for extension to glycan structures of mammalian type and the *in vivo* synthesis of complex N-glycans with terminal *N*-acetylglucosamine residues has been demonstrated in *T. reesei* (Maras et al. 1999).

3.5 Proteolytic Processing in the Secretory Pathway

In addition to folding and glycosylation, protein maturation may involve proteolytic processing that takes place in the ER and the Golgi apparatus. Several eukaryotic proteins contain prosequences (propeptides) that may have an important role in secretion, folding, and organelle targeting [Baker et al. (1993) and Chang et al. (1994); reviewed in Conesa et al.

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(2001) and references therein]. Cleavage of the propeptide is necessary for the activation of a number of eukaryotic enzymes, typically lipases and proteases, including those produced by fungi.

The fact that heterologous proteins originating especially from higher eukaryotes such as mammals and plants are poorly produced in filamentous fungi may indicate a problem in their processing. Therefore, studies into the mechanism and cellular basis for protein processing are necessary for better understanding of the production bottlenecks in heterologous protein expression and secretion.

Proteases that process polypeptides either intracellularly or on cell surfaces feature calcium-dependent serine endoproteases related to subtilisin and kexin families (Henkel et al. 1999; Steiner et al. 1992; van den Ven 1993). Intracellular processing of the majority of propeptides by Kex2p-like proteases occurs at a dibasic cleavage site after Lys–Arg (KR) or Arg–Arg (RR). Dibasic sites that resemble the Kex2p target sites are frequently found in sequences of secretory proteins in filamentous fungi (Calmels et al. 1991; Goller et al. 1998b). Studies with the endogenous *T. reesei* xylanases exhibiting proprotein processing sequences resonance RR ↓ R ↓ A, KR ↓ Q showed that secretion was inhibited by aminophenylmethylsulfonyl fluoride (pAPMSF) which inhibits dibasic endopeptidase activity (Goller et al. 1998b). In addition to Kex2p like proteases, experimental data points to the existence of different, yet unidentified endoproteolytic proprotein processing enzymes in the filamentous fungus *T. reesei* (Nykänen et al. 2002a; Nyyssönen et al. 1993).

In *Aspergillus* spp., a Kex2p cleavage site has been introduced in fusion proteins at the fusion junction to separate the foreign protein from the endogenous carrier [Contreras et al. (1991); reviewed in Gouka et al. (1997) and discussed in Section 2.6]. In a study by Jalving et al. (2000), a kexin-like maturase was characterized and its gene, *kexB*, isolated from *A. niger*. The cloned gene was then used to produce *A. niger* strains either overexpressing or lacking the *kexB* gene. Expression of a glucoamylase-human interleukin-6 fusion protein with an engineered Kex2p cleavage site in the KexB defective strain resulted in inability of the *kexB* disruptant to process the fusion protein at the dibasic target site. Engineering of Kex2p cleavage sites into constructs expressing the catalytic subunit of bovine enterokinase and human mucus inhibitor protein fused to the *glaA* (glucoamylase) has also been shown to result in the correct processing of the fusion protein at the Kex2p site in *A. niger* (Krasevec et al. 2000; Mikosch et al. 1996). In spite of proteolytic processing, the levels of heterologous proteins secreted in the culture medium remained at 3 and 5 mg/l, respectively. Nykänen et al. (2002a) demonstrated that proteolytic processing of the barley cysteine endoproteinase (EPB) could occur by Kex2p-like cleavage at three of the four dibasic sites in the protein sequence and that glycosylation of the heterologous protein interfered with the final processing of the protein by an unknown peptidase. Incomplete processing decreased the recombinant EPB activity. Maturation of heterologous fungal proteins such as the *Hormoconis resinae* glucoamylase (Nykänen 2002b) and *H. grisea* xylanase (Faria et al. 2002) when expressed in *T. reesei* occurred by Kex2p-like processing. Based on the findings so far, it is evident that Kex2p-like cleavage in the trans-Golgi plays an important role in the processing of both homologous and heterologous proteins in filamentous fungi.

3.6 Inner View of a Secreting Hypha

The fate of proteins in the secretory pathway has been followed by using gene fusions to fluorescent proteins such as green fluorescent protein (GFP) [e.g., Gordon et al. (2000) and references therein; Lorang et al. (2001)] and by the application of (ultrastructural) immunoelecronmicroscopy [e.g., Kurzatkowski et al. (1993) and Nykänen et al. (1997; 2002a,b,c)]. The use of metabolic inhibitors such as tunicamycin inhibiting protein glycosylation, and monensin and Brefeldin A affecting vesicle trafficking has provided novel information on the infrastructure of the secretory machinery [reviewed in Nykänen (2002b)]. These studies have confirmed that the fungal secretory machinery shares the main features of the corresponding pathways described for yeast and mammals. Attempts to quantify the amount of a heterologous protein in the secretory organelles have also been carried out (Nykänen et al. 2002a,c). This type of an approach will provide valuable information on the secretion dynamics in the fungal hyphae secreting foreign proteins and for unraveling the bottleneck compartments in the production of complex heterologous proteins.

3.6.1 Genes Involved in the Secretion Process

In order to understand the secretory pathway of filamentous fungi in the hope of eventually being able to improve secretion, the cloning of genes involved in the secretion processes beyond the ER has been attempted although the number of isolated genes is yet limited [reviewed by Conesa et al. (2001) and Vasara (2002)]. A class of proteins of interest are the small GTPases that could have a regulatory role in the vesicle function and the secretion process. No clear results are yet available on improvement of the secretory processes through modulation of expression of these genes although their effect on secretion seems evident. As an example, inactivation of the putative *SEC4* homolog in *A. niger* had temperature-dependent effects on secretion and affected mycelial morphology (Punt et al. 2001).

Recently, two genes encoding general signal transduction molecules RHOIII and 14-3-3, involved in many cellular processes, have been described for *T. reesei* (Vasara et al. 2001; 2002). The genes were cloned by complementation of the *S. cerevisiae* secretory mutant *sec15-1*. Disruption of the RHOIII encoding gene from *T. reesei* did not have any effect on glucose or cellobiose but interestingly, reduced growth and secretion on cellulose-containing medium where production of secreted proteins is high in this fungus.

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3.7 The Fungal Cell Wall

The majority of secreted proteins including heterologous gene products are excreted through the growing hyphal tip (Wessels 1993). However, there are indications that an extremely effectively secreted protein such as the main CBHI in *T. reesei*, can also be excreted by older cell compartments (Nykänen et al. 2002a). Ultrastructural studies have also highlighted the importance of the fungal cell wall as a final barrier of protein excretion. For example, the heterologous calf chymosin secreted by *T. reesei* transformants without fusion to an endogenous carrier has been shown to be trapped in the cell wall (Nykänen et al. 2002c).

A novel approach into identification of fungal cell wall proteins is featured by the study of Lim et al. (2001) where proteins were isolated from the cell envelopes of *T. reesei* and separated using 2-D gel electrophoresis. From the 32 spots of which amino acid sequence was obtained, 20 were identified using publicly available databases. A dominant protein found in mycelia was HEX1, the major protein of the Woronin body (Tenney et al. 2000), a structure unique to filamentous fungi. The fact that 12 proteins for which a clear signal was obtained, could not be identified emphasizes current restrictions in the proteomic approach which relate to the limited information available on the databases. However, together with the exponentially increasing knowledge emerging from fungal genomic studies (discussed briefly in Section 5), identification of a growing number of proteins discovered through proteomic studies is only a matter of time.

3.8 Strains Deficient in Extracellular Proteases

Proteases have a number of important tasks in the fungal organism. In addition to intracellular proteolytic processing discussed in Section 3.5, proteases secreted from filamentous fungi are needed for the hydrolysis of complex substrates to nutrients for the growing fungus. By the same token, production of extracellular proteases may affect the yields of both homologous and heterologous proteins secreted into the culture medium. Especially heterologous proteins can be sensitive to proteolytic degradation.

The portfolio of proteases produced varies according to the fungal species, strain, and growth medium. Therefore, when choosing a suitable strain for protein expression, familiarity with the proteases of the host strain is of considerable advantage. Specific proteinase activities in fungal cell extracts can be studied, for example, by using commercially available fluorescently labeled class-specific peptidyl substrates and specific proteinase inhibitors. Among proteases produced by *A. niger*, acid proteases including aspartyl-, serine-, and carboxyl proteases are dominating [reviewed in van den Hombergh et al. (1997)]. Similar types of proteolytic activities are also produced by *T. reesei* (Bradford 2000).

Protease-deficient strains can be created by classical mutagenesis and screening, recombination, and targeted disruption of protease genes. Classical mutagenesis, involving treatment of fungal conidia with, e.g., UV followed by screening of developing colonies on plates containing casein or skim milk has been effectively employed to *A. niger* (Mattern et al. 1992) and *T. reesei* (Mäntylä et al. 1994). Strains produced in this way have been successfully used as hosts for heterologous protein expression. However, the random mutagenesis approach will not allow for direct generation of strains deficient in a particular proteinase.

Pending on the availability of isolated protease genes, disruption of particular endogenous genes becomes possible. For example, disruption of the gene encoding the major extracellular protease in *A. awamori* resulted in 20% reduction of the extracellular protease compared to the wild type (Berka et al. 1990a,b). In cases where genetic recombination can be used, strains deficient in multiple proteinases can be produced with a relative ease. In addition to manipulating the structural protease genes, genes involved in protease regulation can be addressed (van den Hombergh et al. 1997). Supernatants of a series of different protease mutants can be used to test the sensitivity of a particular heterologous protein to host proteases before committing to transformation.

4 PHYSIOLOGY AND PRODUCTIVITY

Tools for studying fungal morphology in submerged culture have developed rapidly. Image analysis and a selection of commercially available fluorescent probes are currently being used in studies relating fungal morphology to productivity. In combination with fluorescence microscopy and automated image analysis, fluorescent probes have been applied to develop a morphologically structured model to amylase production in *A. oryzae* (Agger et al. 1998). These types of studies and models may provide a guide for monitoring product fermentation and tailoring high performing strains for particular bioprocesses (McIntyre et al. 2001).

Another area developing rapidly with respect to fermentation physiology of both homologous and recombinant gene products is measurement of metabolic fluxes in the fungal cultures in order to determine which pathways are active under particular growth conditions. Such information is a valuable tool for the optimization and regulation of fermentation processes (Pedersen et al. 1999). For example, quantification of metabolic fluxes in the wild type and a recombinant α-amylase producing *A. oryzae* in a carbon limited chemostat culture showed that the fluxes through the pentose phosphate pathway were up to 26% higher for the recombinant strain when compared to the wild type.

4.1 Novel Promoter Strategies

The use of suitable promoters combined with particular growth media provides a strategy for the production of gene products of interest. For example, expression of

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a heterologous gene under the constitutive glycolytic promoter pyruvate kinase A (*pkiA*) on a medium containing high amounts of glucose and ammonium, allows synthesis of the heterologous product under conditions where the production of most of the extracellular proteases is repressed (van den Hombergh et al. 1994; Nakari-Setälä et al. 1993; 1995). Combination of a growth rate correlated promoter together with a growth rate independent promoter also exhibiting different pH optima has allowed prolonged production of a recombinant glucoamylase over the different phases of fermentation of *F. venenatum* (Gordon et al. 2001). The multiple promoter strategy appears attractive and can be applied to other fungi as well where suitable promoters are available.

5 TOWARDS A HOLISTIC VIEW: GENOMIC AND PROTEOMIC APPROACHES

Expression of a particular gene in a fungal host involves a complex genetic network. Such networks can be studied using applied genomics technologies including transcriptional profiling (gene expression), proteomic analysis (protein profiling), metabolomics (metabolite mapping), and computational modeling. The importance of fungi to man has initiated several programs aiming at genomic sequencing. These initiatives have targeted genetically well-known academic fungi such as *Neurospora crassa* (e.g., http.//www.mips.biochem.mpg.de/proj/neurospora/), *P. chrysosporium* (http://www.jgi.doe.gov/programs/whiterot/whiterot_mainpage.html), and *A. nidulans* (Roe et al. 1999), plant pathogens such as *M. grisea* (Martin et al. 2002), opportunistic human pathogens such as *A. fumigatus* (Brookman and Denning 2000) and biotechnically relevant fungi *A. niger* and *T. reesei* (Chambergo et al. 2002). Genomics is also on the agenda to advance microbial food fermentations as well as food safety and functionality (de Vos 2001).

Functional genomics aims to identify the roles of genes present in sequenced genomes. This can be achieved by combining genomic analyses with transcriptional profiling and proteomic analyses to result in a meaningful interpretation of metabolic responses to, e.g., changing growth environment or pathogenesis. While transcription profiling identifies the genes active under particular circumstances, a proteomic display complements the information by showing the forms of actual proteins synthesized from the genes by providing a protein print out. Proteomics can be used, for example, in strain comparisons, to map the proteins in a given host strain used for gene expression and obtain a profile of the proteins from a recombinant strain producing a heterologous gene product.

6 CONCLUSIONS

Modern fungal strain improvement is an integrated approach combining basic biology, molecular strain development, fermentation physiology, and functional genomic approaches in order to produce strains for disciplines ranging from industrial enzyme production to agriculture, sustainable environment, and medicine. Gene expression is influenced, for example, by transcriptional activators and repressors of which the activity is influenced by yet other gene products. Therefore, expression of a particular gene in a fungal host involves a complex genetic network. The soaring technological developments in research tools have made possible to study these networks at the whole cell context. Supported by the exponentially growing bioinformatics such as genome and protein databases amongst many others, a more holistic approach aiming at understanding the critical points of gene expression in filamentous fungi seems achievable.

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The Genetic Improvement of Wine Yeasts

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1 INTRODUCTION

Modern wine production contributes significantly to the economies of many countries. The rapid expansion of wine grape cultivation in the New World wine-producing countries, together with the worldwide decline in alcoholic beverage consumption, however, resulted in an ever-increasing gap between wine production and wine consumption (Bisson et al. 2002; Pretorius and Bauer 2002). Fierce competition for market share has become the driving force behind the enhancement of wine quality, purity, uniqueness, and diversity by developing specialized yeast starter culture strains for specific styles of wine.

Historically, the yeasts found on the surfaces of grapes and winery equipment have been responsible for the conversion of grape juice into wine. In such spontaneous fermentations, there is a sequential development of indigenous yeasts (e.g., *Kloeckera/Hanseniaspora*, *Candida*, *Metschnikowia*, and *Pichia*), with the final stages invariably being dominated by the alcohol-tolerant strains of *Saccharomyces cerevisiae* (Pretorius et al. 1999). This ellipsoidal shaped species that reproduces asexually through budding (mitotic division), or sexually by sporulation (meiotic segregation and haploidization) and mating (diploidization), has therefore become known as the "wine yeast." During the second half of the last century, however, increased understanding of the diversity, physiology, and genetics of *S. cerevisiae* strains led to the selection of starter culture strains on the basis of their fermentation behavior. This led to significant improvements in the control of fermentation and the quality of the end product. It has become common practice to inoculate grape juice with a specific active dried wine yeast starter culture strain to produce a predetermined style of wine.

There are many ways in which *S. cerevisiae* can be genetically improved. Some techniques alter limited regions of the genome while others are used to recombine or rearrange the entire genome. Combining classical breeding techniques and recombinant DNA methods has dramatically increased the genetic diversity that can be introduced into yeast cells (Pretorius 2000). Since the announcement of the complete nucleotide sequence of the 13000-kb genome, (distributed along 16 linear chromosomes carrying approximately 6000 protein-encoding genes) of a laboratory strain of *S. cerevisiae* in 1996, and the recent development of technologies with which its entire genome, transcriptome, proteome, and possibly metabolome can be analyzed (Goffeau et al. 1996; Oliver 1996), considerable progress has been made in the development of new designer wine yeast strains that meet current demands of both wine producers and consumers (Dequin 2001; Henschke 1997; Pretorius 1999; 2000; 2001; Pretorius and Bauer 2002; Pretorius and Van der Westhuizen 1991; Querol and Ramon 1996; Rainieri and Pretorius 2000; Vivier and Pretorius 2002).

This Chapter is based on a recent, comprehensive review of the tailoring of wine yeasts by Pretorius (2000) and highlights the application of gene technology to wine yeast strains, the current status of genetically improved wine yeasts, and potential targets for further strain development.

2 THE DEVELOMENT OF YEASTS FOR THE IMPROVEMENT OF THE EFFICIENCY OF FERMENTATION

The most important selection criteria in strain development programs relate to achieving a better than 98% conversion of grape sugar to alcohol and carbon dioxide. This process must take place at a controlled rate and without the development of off-flavors (Henschke 1997). However, the growth and fermentation properties of wine yeasts have not yet been defined genetically. The genetic definition of these attributes is made even more complex by the fact that lag phase, rate and

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efficiency of sugar conversion, resistance to inhibitory substances, and total time of fermentation are strongly affected by the physiological condition of the yeast, and also by the physicochemical and nutrient properties of the grape must (Henschke 1997).

The predictability of fermentation and the quality of the wine are directly dependent on wine yeast attributes that assist in the rapid establishment of numerical and metabolic dominance in the early phase of wine fermentation, and that determine the ability to conduct an even and efficient fermentation with a desirable residual sugar level. Many factors affect the fermentation performance of wine yeasts. General targets for the improvement of fermentation performance are increased resilience and stress resistance of active dried yeast cells, improved grape sugar and nitrogen uptake and assimilation, enhanced resistance to ethanol and other microbial metabolites and toxins, resistance to heavy metals and agrochemical residues, and tolerance to sulfite.

2.1 Improved Resilience and Stress Tolerance

The terms *resilience*, *stress tolerance*, *fitness*, and *vigor* of wine yeast starter culture strains all relate to the maintenance of both cell viability (the relative proportion of living cells within a population) and vitality (the measure of metabolic activity) during the process of yeast manufacturing and wine fermentation (Walker 1998). Yeast strains differ from one another in terms of their *inherent* biological resilience. A particular yeast strain's resistance to stress is not only determined genetically, but also exposure to a mild stress can result in improved resistance to subsequent exposures, either to more extreme forms of the same stress or to other stresses. These phenomena are referred to as *acquired stress resistance* and *cross protection* (Siderius and Mager 1997). The choice of an inherently stress-tolerant strain, as well as the specific preparation procedure used during the production process of commercially active dried wine yeast starter cultures, therefore can affect the degree of viability and vitality, as well as the subsequent fermentation performance of the cultures (Bauer and Pretorius 2000). The inherent stress tolerance of specific strains and the frequency with which stuck or sluggish fermentation occurs are inversely correlated (Ivorra et al. 1999). Stress conditions lead to a reduction in the speed of growth and the survival rate, thereby reducing fermentation efficiency. However, the better and faster a yeast strain is able to adapt to changes in the environment, the faster the completion of fermentation.

Successful adaptations by the yeast cells to changes in extracellular parameters during the yeast manufacturing process and wine fermentation require the timely perception (sensing) of chemical or physical environmental parameters, followed by accurate transmission of the information to the relevant compartments of the cell. Chemical signals emanating during the manufacture of active dried wine yeast cultures and during wine fermentations include the availability/concentration of water and certain nutrients (e.g., fermentable sugars, assimilable nitrogen, oxygen, vitamins, minerals, ergosterol, and unsaturated fatty acids) and the presence of inhibitory substances (e.g., ethanol, acetic acid, fatty acids, sulfite, agrochemical residues, and killer toxins). Signals of a physical nature include temperature, pH, agitation, and osmotic pressure. For example, physiological and morphological modifications in response to a limited supply of essential nutrients, such as carbon and nitrogen, include a shift in transcription patterns, modification of the cell cycle, a change in budding pattern and strongly polarized growth (Gagiano et al. 2002). It is very clear that a complex network of interconnected and cross-talking signal transduction pathways, which relies on a limited number of signaling modules, governs the required adaptive responses to changes that occur when yeast strains are manufactured and during wine fermentations (Bauer and Pretorius 1999; 2000). The yeast cell's various stress-signaling pathways, which ensure that it can timely implement appropriate responses to hyperosmotic conditions, nutrient limitation and depletion, ethanol toxicity, suboptimal temperatures, high levels of carbon dioxide, and other stress factors, are discussed in depth by Bauer and Pretorius (2000).

It is possible for the manufacturers of active dried wine yeast starter cultures to positively influence the degree of viability and vitality, as well as the subsequent fermentation performance of their cultures, by the way the yeasts are cultivated (Degré 1993). Industrial cultivation of wine yeasts can have a profound effect on the microbiological quality, fermentation rate, production of hydrogen sulfide, ethanol yield and tolerance, resistance to sulfur dioxide as well as tolerance to drying, and rehydration. As a result of the roles played by trehalose and glycogen in a yeast cell's response to variations in environmental conditions, it generally is recommended that the manufacturers of active dried wine yeast starter cultures cultivate their yeast in such a way that the maximum amount of these storage carbohydrates is accumulated in the yeast cells.

In *S. cerevisiae*, trehalose is associated with nutrient-induced control of cell cycle progression; sporulation, growth resumption and growth rate, control of glucose sensing, transport, and the initial stages of glucose metabolism; as well as stress protection against dehydration, freezing, heating and osmostress, nutrient starvation and toxic chemicals such as ethanol, oxygen radicals, and heavy metals. The trehalose content of the yeast cell is probably one of the most important factors affecting the resistance of yeasts to drying and subsequent rehydration (Degré 1993). The fact that this disaccharide is accumulated on both sides of the plasma membrane is thought to confer stress protection by stabilizing the membrane structure of the yeast. Trehalose is synthesized from glucose-6-phosphate and UDP-glucose by the *TPS*1-encoded trehalose-6-phosphate synthetase and converted to trehalose by the *TPS*2-encoded trehalose-6-phosphate phosphatase (Francois et al. 1997). cAMP-dependent phosphorylation mechanisms mediate the

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regulation of trehalose synthesis and degradation (by the *ATH*1-encoded trehalase) (Thevelein and De Winde 1999).

Glycogen, another carbohydrate reserve whose accumulation by yeast propagated for drying has been linked to enhanced viability and vitality upon reactivation, provides a readily mobilizable carbon and energy source during the adaptation phase. Glycogen is biosynthesized by glycogen synthase, which catalyzes the sequential addition of glucose from UDP-glucose to a polysaccharide acceptor in a linear α-1,4 linkage, while branching enzymes are responsible for the formation of α-1,6 branches (Walker 1998). Two forms of glycogen synthase are present in *S. cerevisiae*, namely Gsy1p and Gsy2p. The *GSY*1 gene is expressed constitutively at a low level along with growth on glucose, while the level of the *GSY*2-encoded glycogen synthase increases at the end of the exponential phase of growth, when glycogen accumulates (Francois et al. 1997). *GSY*2 therefore encodes the major glycogen synthase. Glycogen breakdown, which is catalyzed by glycogen phosphorylase immediately following the depletion of nutrients at the end of fermentation, is accompanied by sterol formation (Francois et al. 1997). Sterol is essential for yeast vitality, which means that low levels of accumulated glycogen in active dried wine yeast starter cultures may result in insufficient yeast sterols. This, in turn, may impair yeast performance on inoculation into grape juice (Walker 1998).

As trehalose and glycogen fulfil multiple roles in increasing the survival of *S. cerevisiae* cells exposed to several physical and chemical stresses, they have important implications for the resilience and stress tolerance of active dried wine yeast starter cultures upon reactivation. There is a strong incentive to develop wine yeast strains with a superior ability to accumulate trehalose and glycogen. However, due to the complex stress response mechanisms in yeast, it is not yet clear whether the modification of the expression levels of the *TPS*1, *TPS*2, *GSY1, GSY2, SUT*1, and/or *SUT*2 genes would contribute to the yeast fitness and fermentation performance of starter culture strains. For example, attempts to increase stress resistance through the overexpression of the *TPS*1 and *TPS*2 genes have not yet established a convincing linear link between trehalose content and stress resistance (Bauer and Pretorius 2000). On the other hand, it has been shown that a yeast strain deleted for the *ATH*1 trehalase gene contained higher levels of trehalose and showed improved survival after dehydration, freezing, and ethanol shock (Kim et al. 1996; Shima et al. 1999). The overexpression of two aquaporin genes (*AQY*1 and *AQY*2) recently was shown to enhance significantly the freeze resistance of baker's yeasts (Tanghe et al. 2003; Teunissen et al. 2003; Van Dijck et al. 2000). This might provide new clues regarding the enhancement of stress resistance in wine yeasts.

The main reason for the relatively slow progress in the improvement of resilience in wine yeast strains is the intrinsic complexity of the interwoven network of pathways and mechanisms involved in stress resistance. However, it is expected that the systematic analysis of the genome, transcriptome, and proteome soon will lead to the elucidation of these stress response pathways. The first data based on such systematic approaches were published recently (Backhus et al. 2001).

2.2 Improved Efficiency of Sugar Utilization

The main sugars, glucose and fructose, present in the grape must in *S. cerevisiae*, are metabolized via the glycolytic pathway. Many attempts have been made to increase the rate of glycolytic flux. The first step to ensure efficient utilization of grape sugar by wine yeasts is to replace any nonfunctional mutant alleles of genes encoding the key glycolytic enzymes. Various control points have been suggested, including the reactions catalyzed by the hexokinase, phosphofructo-kinase and pyruvate kinase. Contrary to the common assumption at the time, it was demonstrated that the overexpression of a combination of the genes encoding these glycolytic enzymes has no effect on the rate of glycolytic flux (Schaaff et al. 1989). A second hypothesis was based on the supposition that a decrease in the intracellular ATP concentration would lead to an increase in the rate at which CO_2 was produced. This was investigated by the derepression of the ATP-consuming gluconeogenic enzymes, fructose-1,6-diphosphatase, and phosphoenolpyruvate carboxykinase (Navas et al. 1993). Despite the absence of evidence for a functional futile cycling, the derepressed strain showed a 25% increase in the rate of glucose consumption, but also a significant reduction in biomass yield. Another attempt to increase the glycolytic flux involved overexpression of the genes encoding seven enzymes involved in the latter stages of glycolysis. This strategy led to an increased glycolytic flux, but only under conditions of increased ATP demand (Smits et al. 2000).

The limited success of the above-mentioned metabolic engineering indicated that the enzymatic steps in the glycolytic pathway did not appear to be rate limiting. This refocused the attention on sugar uptake as a major control site for the rate of glycolytic flux under anaerobic conditions. The hypothesis is that the rate of alcohol production by wine yeast is limited primarily by the rate of glucose and fructose uptake. In winemaking, therefore, the loss of hexose transport toward the end of fermentation may result in wine with reduced alcohol yields and higher levels of residual sugars (Walker 1998). Since *S. cerevisiae* is a glucophilic yeast, fructose (the sweetest of all sugars) is always present in a larger amount than glucose in bottled wine. In the case of sluggish or stuck fermentations, the preference of wine yeasts for glucose over fructose can lead to excessive residual fructose levels that compromise the quality of the wine.

There are three ways in which sugars can enter yeast cells: simple net diffusion, facilitated (carrier-mediated) diffusion, and active (energy-dependent) transport. In grape-must fermentations, in which high sugar concentrations are common, free diffusion may account for a very small proportion of sugar uptake into yeast cells. Various complex

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mechanisms are required for efficient translocation of glucose, fructose, and other minor grape sugars into the cell, because the plasma membranes of yeast cells are not freely permeable to polar sugar molecules. The hexose transporter family of *S. cerevisiae* consists of more than 20 proteins, comprising high, intermediate, and low affinity transporters and at least two glucose sensors (Pretorius 2000). Many factors affect both the abundance and intrinsic affinities for hexoses of these transporters that are present in the plasma membrane of wine yeast cells. Among these factors are glucose concentration, growth stage and rate, presence or absence of molecular oxygen, rate of flux through the glycolytic pathway, and nutrient availability (particularly of nitrogen) (Boulton et al. 1996).

The precise mechanisms and regulation of grape-sugar transport of wine yeast are still unclear. However, it is well known that glucose uptake occurs rapidly down a concentration gradient, reaches equilibrium and is therefore not accumulative (Ciriacy and Reifenberger 1997). Several specific, energy-dependent glucose carriers mediate the process of facilitated diffusion of glucose and proton symport is not involved. Phosphorylation by the *HXK*1- and *HXK*2-encoded hexokinases and the *GLK*1-encoded glucokinase is linked to high-affinity glucose uptake. Glucose transporters, encoded by *HXT*1–*HXT*18 and *SNF*3, are stereospecific and will translocate only glucose, fructose, and mannose. Some members of this multigene permease family affect the uptake of glucose, galactose, glucose, and mannose, or that of glucose, fructose, and galactose, but none has yet been described that specifically affects fructose uptake (Walker 1998). It appears that fructose is transported via facilitated diffusion rather than active transport in *S. cerevisiae*, whereas related species *S. bayanus* and *S. pastorianus* possess fructose–proton symporters.

There recently has been a spectacular increase in the amount of information on sugar sensing and the entry of sugar into yeast cells, and several laboratories have identified this main point of control of glycolytic flux as one of the key targets for the improvement of wine yeasts. For example, certain members of the *HXT* permease gene family are being overexpressed in an effort to enhance sugar uptake, thereby improving the fermentative performance of wine yeast strains. There is anecdotal evidence that the overexpression of *HXT*1, which encodes the hexokinase with the highest affinity for fructose (but still a significantly lower affinity than for glucose) among the native *S. cerevisiae* hexokinases, has resulted in improved fructose utilization during fermentation trials. Unconfirmed data also indicate that the overexpression in *S. cerevisiae* of the *S. pastorianus FSY*1 gene, encoding a fructose/H^+ symporter, could lead to improved fructose uptake during wine fermentations. However, greater detail is required about the complex regulation of glucose and fructose uptake and about glycolysis as it occurs in grape juice before novel strategies can be devised to improve the fermentation performance of wine yeast and to prevent sluggish or stuck fermentations.

2.3 Improved Nitrogen Assimilation

Nitrogen is quantitatively second only to carbon of all the nutrients assimilated by yeast during wine fermentations (Henschke and Jiranek 1993). Carbon–nitrogen imbalances, specifically deficiencies in the supply of assimilable nitrogenous compounds, remain the most common causes of poor fermentative performance and sluggish or stuck fermentations (Jiranek et al. 1995b). Problematic and incomplete fermentations occur because nitrogen depletion irreversibly arrests hexose transport. Further problems related to the nitrogen composition of grape must include the formation of reduced-sulfur compounds, in particular hydrogen sulfide, and the potential formation of ethyl carbamate from metabolically produced urea (Henschke and Jiranek 1993).

Grape sugars are usually present in excess (often exceeding 20% *w*/*v*) of what is needed for maximal yeast growth. In contrast, the total nitrogen content of grape juices ranges 40-fold from 60 to 2400 mg/l and can therefore limit growth (Henschke and Jiranek 1993). The assimilable content of grape must is dependent upon grape cultivar and rootstock, as well as several aspects of vineyard management, including nitrogen fertilization, berry maturation, vine water status, soil type and fungal infection (Henschke and Jiranek 1993). If the nitrogen level of a grape juice is below 150 mg/l, it most likely will become a problem ferment due to inadequate yeast growth and poor fermentative activity. Nitrogen deficiency can be prevented by optimizing vineyard fertilization and supplementation with ammonium salts such as diammonium phosphate (DAP). However, injudicious supplementation with DAP often contravenes the wine industry's desire to minimize its use of additives while producing wines of high quality. In addition, excessive addition of inorganic nitrogen can result in excessive levels of residual nitrogen, leading to microbial instability and ethyl carbamate (and phosphate in the case of DAP) accumulation in wine (Jiranek et al. 1995a–c). As a result, the degree of supplementation of inorganic nitrogen in grape juice is strictly regulated. The major nitrogenous compounds in the average grape must are proline, arginine, alanine, glutamate, glutamine, serine, and threonine (Boulton et al. 1996). Ammonium ion levels may also be high, depending on the grape variety and vineyard management. Proline and arginine account for 30–65% of the total amino acid content of grape juices. Proline accumulation at high levels in grape must is associated with grapevine stress, in particular with low moisture, whereas high levels of γ-aminobutyrate, another nitrogen compound, may be formed in the grape berries, probably postharvest and prior to processing the grapes (Boulton et al. 1996).

Saccharomyces cerevisiae cannot hydrolyze grape proteins adequately to supplement nitrogen-deficient musts, and it therefore relies on the ammonium and amino acids present in the juice. Wine yeasts can distinguish between readily and poorly used nitrogen sources. The preferred nitrogen source is ammonium and, as it is consumed, the amino acids are taken

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up in a pattern determined by their concentration relative to the yeast's requirements for biosynthesis and to total nitrogen availability (Salmon and Barre 1998). The genes involved in the uptake and catabolism of poorly utilized nitrogen sources (including proline) are repressed when readily used nitrogen, such as ammonium, glutamine, or asparagine, is present. This nitrogen catabolite repression exerted upon nonpreferred nitrogenous compounds rigorously impairs the assimilation of proline and arginine, as both amino acids depend on the proline utilization pathway (Salmon and Barre 1998). The proline content of wine is generally not less than that of grape juice, and it therefore appears that proline is not taken up by wine yeast under anaerobic fermentative conditions (Boulton et al. 1996). Proline is transported into *S. cerevisiae* by the general amino acid permease and the *PUT*4-encoded proline-specific permease. Once inside the yeast cell, the *PUT*1-encoded proline oxidase and *PUT*2-encoded pyrroline-5-carboxylate dehydrogenase convert the proline to glutamate in the mitochondria. The *PUT*3-encoded activator and the *URE*2-encoded repressor regulate the expression of both *PUT*1 and *PUT*2. Ure2p represses the transcription of *PUT*1 and *PUT*2 under nitrogen-limiting conditions, while the *GLN*3-encoded regulator has no effect on these genes (Salmon and Barre 1998).

Wine yeast strains vary widely in their nitrogen requirement, which means that an obvious target for strain improvement is to select or develop starter strains that are more nitrogen efficient for use in low-nitrogen musts (Henschke 1997; Jiranek et al. 1995a–c). A mutant containing a *ure*2 recessive allele was constructed in an effort to develop wine yeast strains that are relieved from nitrogen catabolite repression and are capable of utilizing proline more efficiently under winemaking conditions (Salmon and Barre 1998). This mutation deregulates the proline utilization pathway, thereby improving the overall fermentation performance of the *ure*2-carrying yeast. This could be the first step towards the development of wine yeasts that can efficiently assimilate the abundant supply of proline in grape juice under fermentative conditions.

2.4 Improved Ethanol Tolerance

The dilemma facing the winemaker is that, while ethyl alcohol is the major desired metabolic product of grape-juice fermentation, it is also a potent chemical stress factor that often underlies sluggish or stuck fermentations. Apart from the fact that an excessive sugar content inhibits yeast growth and fermentation, the production of excessive amounts of ethanol as a result of harvesting overripe grapes is known to inhibit the uptake of solutes (e.g., sugars and amino acids) and to inhibit yeast growth rate, viability, and fermentation capacity (Boulton et al. 1996).

The physiological basis of ethanol toxicity is complex and not well understood. It appears that ethanol impacts mainly on membrane structural integrity and membrane permeability (Boulton et al. 1996). The primary sites of ethanol action include the yeast cell's plasma membrane, hydrophobic proteins of mitochondrial membranes, nuclear membrane, vacuolar membrane, endoplasmic reticulum, and cytosolic hydrophilic proteins (Walker 1998). Increased membrane fluidity and permeability due to ethanol challenge seem to result in a futile cycling of protons and dissipation of ATP energy. The dissipation of the proton gradient across the membrane and ATP is not only affected by increased permeability to protons, but ethanol may also directly affect the expression of the ATPase-encoding genes (*PMA*1 and *PMA*2) and membrane ATPase activity (Henschke 1997). This provides an explanation for the interference of ethanol with energy-coupled solute transport in yeast cells.

A number of intrinsic and environmental factors are known to synergistically enhance the inhibitory effects of ethanol. Amongst these are high fermentation temperatures, nutrient limitation (especially oxygen, nitrogen, lipids, and magnesium ions), and metabolic byproducts, such as other alcohols, aldehydes, esters, organic acids (especially octanoic and decanoic acids), certain fatty acids, and carbonyl and phenolic compounds (Edwards et al. 1990). If the physicochemical environment during the cultivation and manufacturing of active dried wine yeast starter cultures and during the actual vinification process is manipulated, the self-protective adaptations of the yeast cells can be promoted. If the yeast cells are exposed to ethanol in advance (physiological preconditioning), adaptive stress responses are elicited that confer a degree of resistance to subsequent exposure to high levels of ethanol. Osmotic pressure, media composition, modes of substrate feeding, and byproduct formation also play important roles in dictating how yeast cells tolerate ethanol during vinification (Walker 1998). Most of the so-called survival factors (e.g., certain unsaturated long-chain fatty acids and sterols) are formed only in the presence of molecular oxygen. This helps to explain the great success achieved with the use of commercial starter cultures that are cultivated under highly aerobic conditions and in low glucose concentrations. These starter yeast cells contain high levels of survival factors that can be passed on to their progeny during the six or seven generations of growth in a typical wine fermentation (Boulton et al. 1996).

Wine yeast strains usually contain higher levels of survival factors than nonwine *Saccharomyces* strains (Boulton et al. 1996). Wine yeast also displays a greater physiological response to ethanol challenge than the nonwine strains. These defensive adaptations of wine yeasts confer enhanced ethanol tolerance and range from alterations in membrane fluidity to the synthesis of detoxification enzymes. Responses include a decrease in membrane saturated fatty acids (e.g., palmitic acid); an increase in membrane unsaturated long-chain fatty acids (e.g., oleic acid); phosphatidylinositol biosynthesis (thereby increasing the phospholipid protein ratio in the membrane); elevated levels of cellular trehalose that neutralize the membrane-damaging effects of ethanol; stimulation of stress-protein biosynthesis; enhanced mitochondrial superoxide dismutase activity that counteracts ethanol-induced free radical synthesis; and increased

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synthesis of cytochrome P450, alcohol dehydrogenase activity, and ethanol metabolism (Boulton et al. 1996; Walker 1998).

The genetics of ethanol tolerance are polyvalent and very complex with more than 250 genes and are implicated in the control of ethanol tolerance in yeast (Pretorius 2000). However, some reports claim that the selection of viable mutants with improved ethanol tolerance and fermentation capabilities was enabled by the continuous culture of yeasts in a feedback system in which the ethanol was controlled by the rate of carbon dioxide evolution (Brown and Oliver 1982). Dramatic increases in ethanol tolerance, however, seem to elude researchers. For the time being, therefore, ethanol tolerance in wine yeasts will be addressed by "cell engineering" rather than "genetic engineering."

2.5 Increased Tolerance to Antimicrobial Compounds

In addition to the various yeast metabolites that can interfere with efficient grape-must fermentations, such as alcohols, acetic acid, and medium chain length fatty acids (e.g., decanoic acid), several other antimicrobial compounds can impede the fermentation performance of wine yeasts. These compounds include zymocins (killer toxins), chemical preservatives (especially sulfite), and agrochemicals containing heavy metals (e.g., copper). As *S. cerevisiae* strains vary widely in their ability to resist or tolerate these compounds, the differences may lend themselves as targets for strain development.

2.5.1 Zymocins

The killer phenomenon is widespread among grape, must and wine-related yeast genera, including *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Pichia*, and *Rhodutorula* (Shimizu 1993). The killers themselves, however, are immune to these mycovirus-associated toxins. It is still a matter of controversy whether the growth and zymocidal activity of some wild killer yeasts have the potential to delay the onset of fermentation, cause sluggish or stuck fermentations, and produce wines with increased levels of acetaldehyde, lactic acid, acetic acid, and other undesirable sensory qualities (Shimizu 1993). However, it appears that under certain conditions that favor the development of killer yeast contamination of grape juice (e.g., inefficient inoculation with highly sensitive starter cultures in low-nitrogen musts), potent zymocidal yeasts may indeed contribute to incomplete fermentations. While zymocidal toxins produced by killer strains (K_1, K_2, K_3, K_{28}) of *S. cerevisiae* are lethal only to sensitive strains of the same species, those produced by non-*Saccharomyces* killer species (K_4–K_{11}) may be toxic to a wider range of wine yeast strains and to other wild yeasts. The two *S. cerevisiae* killer toxins that function at wine pH, K_2, and K_{28}, kill sensitive wine yeasts via two different mechanisms: the K_2 toxin acts as an ionophore affecting membrane permeability and the leakage of protons, potassium cations, ATP, and amino acids, whereas the K_{28} toxin inhibits DNA synthesis (Schmitt et al. 1996).

As a consequence of ignorance regarding the role of killer yeasts in wine fermentations, some winemakers use cocultures to inoculate fermentations, one strain being a killer and the other a sensitive strain. The advantage of using killer or neutral wine yeasts should not be underestimated, however. The aim of many strain development programs is to incorporate the mycoviruses from killer yeasts into commercial wine strains via cytoplasmic fusion and cytoduction, or by crossing a haploid derived from a killer wine yeast with haploid cells or ascospores from a sensitive wine yeast (Van der Westhuizen and Pretorius 1992). An alternative to the use of cytoduction and hybridization to develop broad spectrum zymocidal resistance in wine yeasts would be to clone and introduce the toxin-immunity genes from non-*Saccharomyces* killer yeasts into wine yeasts.

2.5.2 Chemical Preservatives

Sulfur dioxide is used extensively in wineries to suppress the growth of unwanted microbes, and tolerance to sulfite forms the basis of selective implantation of active dried wine yeast starter cultures into grape must (Henschke 1997). Membrane transport of sulfite in wine yeasts is by simple diffusion of free sulfur dioxide that dissociates within the cell to SO_3^{2-} and HSO_3^- (Walker 1998). The resulting decline in intracellular pH forms the basis of the inhibitory action. Even though *S. cerevisiae* tolerates much higher levels of sulfite than of most unwanted yeasts and bacteria, excessive SO_2 dosages may cause sluggish or stuck fermentations (Boulton et al. 1996). Wine yeasts differ considerably in their tolerance of sulfite. The underlying mechanism of tolerance and the genetic basis for resistance are still unclear, but once these have been defined better, it may be advantageous to engineer wine yeast starter strains with elevated SO_2 tolerance.

2.5.3 Agrochemicals

The improper application of copper-containing fungal pesticides (copper oxychloride) to control downy mildew (*Plasmopara viticola*) and, to a lesser extent, dead arm (*Phomopsis viticola*) and anthracnose (*Gloeosporium ampelophagum*) could result in copper residues in musts, leading to lagging fermentation and a detrimental effect on wine quality (Tromp and De Klerk 1988). Copper toxicity towards wine yeast cells involves the disruption of plasma membrane integrity and maybe even intracellular interaction between copper, nucleic acids, and enzymes (Avery et al. 1996). Several strategies for copper uptake, efflux, and chelation have been developed by yeasts to control copper-ion homeostasis. One of these protective mechanisms involves the sequestration of copper by the *CUP*1-encoded copper-binding protein, copper–chelatin. These metallothein proteins generally are synthesized when *S. cerevisiae* cells are exposed to potentially lethal levels of toxic metals.

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The copper resistance level of a given yeast strain correlates directly with the *CUP*1 copy number (Fogel et al. 1983). Wine yeasts with resistance to copper could possibly be engineered by cloning and integrating the *CUP*1 gene into their genomes at multiple sites (Henderson et al. 1985). The wine yeast would then be able to tolerate higher concentrations of copper residues in musts. However, copper-resistant wine yeasts should not be used to encourage disrespect for recommended fungicide withholding periods.

3 THE DEVELOPMENT OF YEASTS FOR THE IMPROVEMENT OF THE EFFICIENCY OF WINE PROCESSING

The main aim of fining and clarification during wine processing is the achievement of clarity to ensure the physicochemical stability of the end product. Fining of wine entails the deliberate addition of an adsorptive compound, followed by settling or precipitation of the partially soluble components in wine (Boulton et al. 1996). Sedimentation and racking, centrifugation, or filtration usually achieves further clarification. Clarification generally is thought to produce insignificant compositional changes, whereas fining brings about changes that will prevent further precipitation. As a result, fining can be used to modify the sensory attributes of wine, even though existing clarity may not be a problem.

3.1 Improved Protein Clarification

The gradual development of protein hazes in white wine is considered the next most common physical instability after the precipitation of potassium bitartrate. Protein haze after bottling and shelf storage is induced by high ethanol and low pH. Protein haze is not dependent upon the total protein content, but rather upon specific grape-derived proteins, whose size or isoelectric properties make them very susceptible to solubility limitations (Boulton et al. 1996). Protein instability is thought to be associated with pathogenesis-related (PR) proteins produced in grape berries exposed to fungal attack (Pretorius 2000). Although bentonite treatment can be used to effectively remove these haze-forming proteins, the nonspecific nature of this diatomaceous clay can result in the loss of important aroma and flavor compounds, and the alteration of the sensory characteristics of the wine (Van Rensburg and Pretorius 2000). In addition, bentonite fining is an expensive and laborious practice that generates large volumes of lees for disposal and causes a 5–20% loss of wine (Canal-Llauberes 1993).

One way to omit the bentonite treatment is to apply an appropriate acid protease to hydrolyze the grape PR proteins. So far, however, fungal and yeast enzymes that could degrade these haze-forming proteins have not been found. The vacuolar protease A, encoded by the *PEP*4 gene, is the only *S. cerevisiae* protease that is active at wine pH. Due to the action of proteinase A during yeast autolysis, prolonged storage of wine on the lees after the completion of the alcoholic fermentation renders a wine more protein stable. This has encouraged efforts to develop a white wine yeast strain that secretes protease A into the external medium (Pretorius 2000). However, no extracellular protease activity was detected upon replacing the *PEP*4-encoded vacuolar localization signal with the secretion signal of the yeast *MFα*1-encoded mating pheromone α-factor. Protease A is secreted into the culture medium when the *PEP*4 gene is overexpressed under control of the constitutive yeast alcohol dehydrogenase I gene (*ADH*1) promoter and terminator signals. The overexpression of the *PEP*4 gene under control of this strong promoter in *S. cerevisiae* seems to saturate the vacuolar targeting machinery, thereby directing the excess amount of protease A to the external environment (Van Rensburg and Pretorius 2000).

Bentonite fining is unlikely to be replaced by the addition of proteolytic enzymes to the wine or by engineering a proteolytic wine yeast, due to the inherent resistance of the haze-forming proteins in wine to proteolysis (Pretorius 2000).

3.2 Improved Polysaccharide Clarification

Polysaccharides also influence the clarification and stabilization of must and wine. Polysaccharides originate in the grape itself, from the fungi on the grape and from the microorganisms present during winemaking. The primary polysaccharides causing turbidity, viscosity, and filter stoppages are pectins, glucans (a component of cellulose) and, to a lesser extent, xylans (a component of hemicellulose). Grape pectic substances are heteroplosaccharides, consisting of partially methylated α-1,4-D-galacturonan chains linked to L-rhamnopyranose units carrying neutral side chains (Pretorius 1997). Glucans, such as β-1,3-1,6-glucan produced by the gray mold *Botrytis cinerea* in botrytized grape juice, comprise β-D-glucopyranose units with a high degree of polymerization (Pretorius 1997). Xylans are complex polymers consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains (Pretorius 1997). The enzymatic breakdown of pectic polymers is caused by the de-esterifying action of pectinesterase, which releases the methyl ester groups of the pectin molecule, and by the hydrolase or lyase action of the depolymerases (pectin lyase, pectate lyase, and polygalacturonase), which splits the α-1,4-glycosidic linkages in the polygalacturonate chain. Glucans are hydrolyzed by endoglucanases (β-1,4-D-glucan glucanohydrolase), exoglucanases (β-1,4-D-glucan cellobiohydrolase), cellodextrinases, and cellobiases (β-1,4-D-glucoside glucohydrolase, a member of the β-glucosidase family). The enzymatic degradation of xylans is catalyzed by the synergistic actions of endo-β-1,4-D-xylanases, β-D-xylosidases and α-L-arabinofuranosidases (Pretorius 1997).

The endogenous pectinase, glucanase, xylanase, and arabinofuranosidase activities of grapes and yeasts are not

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sufficient under winemaking conditions to prevent polysaccharide hazes and filter stoppages (Pretorius 2000). Low pH, low temperature, high levels of sulfur dioxide, tannins, and alcohol limit the action of grape-derived polysaccharide-degrading enzymes (Van Rensburg and Pretorius 2000). Therefore, industrial enzyme preparations derived from fungi (*Aspergillus*, *Rhizopus*, and *Trichoderma*), are better adapted for winemaking conditions. Commercial pectinase and glucanase preparations are added as early as crushing to increase juice extraction, or to finished wine to improve filterability (Van Rensburg and Pretorius 2000). Other benefits of the addition of enzyme preparations include the improvement of color extraction in red wine and must quality in terms of settling, fermentability, and aromatic intensity (Ribéreau-Gayon et al. 2000).

However, the addition of commercial enzyme preparations is expensive, and some researchers therefore are looking at the native pectinases and glucanases of *S. cerevisiae*. Certain strains of *S. cerevisiae* were reported to produce pectinesterase, polygalacturonase, and pectin lyase (Gainvors et al. 1994), while all strains of *S. cerevisiae* show some form of glucanase activity (Pretorius 1997). These pectinase and glucanase genes have all been cloned and characterized. The *PGU*1 gene (also known as *PGL*1) codes for a polygalacturonase (Gognies et al. 1999). The *EXG*1 (*BGL*1) gene encodes a protein whose differential glycosylation accounts for the two main extracellular exo-β-1,3-glucanases (EXGI and EXGII), while *EXG*2 encodes a minor exo-β-1,3-glucanase (EXGIII). *BGL*2 encodes a cell wall-associated endo-β-1,3-glucanase, while *SSG*1 (*SPR*1) codes for a sporulation-specific exo-β-1,3-glucanase.

Since the endogenous pectinolytic and glucanolytic activities of *S. cerevisiae* are not sufficient to avoid clarification and filtration problems a wide variety of heterologous pectinase, glucanase, xylanase, and arabinofuranosidase genes have therefore been expressed in *S. cerevisiae*. A pectinolytic wine yeast was developed by coexpressing the *Erwinia chrysanthemi* pectate lyase gene cassette (*PEL5*) and the *Erwinia carotovora* polygalacturonase gene cassette (*PEH*1) in *S. cerevisiae* (Laing and Pretorius 1992; 1993a, b). Likewise, glucanolytic wine yeasts have been developed by expressing glucanase genes in *S. cerevisiae*. These gene cassettes consisted of the *Butyrivibrio fibrisolvens* endo-β-1,4-glucanase gene (*END*1), the *Bacillus subtilis* endo-β-1,3-1,4-glucanase gene (*BEG*1), the *Ruminococcus flavefaciens* cellodextrinase gene (*CEL*1), the *Phanerochaeta chrysosporium* cellobiohydrolase gene (*CBH*1) and the *Saccharomycopsis fibuligera* cellobiase gene (*BGL*1) (Peterson et al. 1998; Van Rensburg and Pretorius 2000). When these glucanase gene cassettes were introduced, *S. cerevisiae* transformants could degrade glucans efficiently. Xylanolytic yeasts were developed by expressing the following gene cassettes in *S. cerevisiae:* the endo-β-xylanase genes from *Aspergillus kawachii* (*XYN*1; Crous et al. 1995), *Aspergillus nidulans* (*xlnA* and *xlnB*; Pérez-González et al. 1996), *Aspergillus niger* (*XYN*4 and *XYN*5; Luttig et al. 1997) and *Trichoderma reesei* (*XYN*2; La Grange et al. 1996; 2001), as well as the xylosidase genes from *A. niger* (*xlnB*) and *Bacillus pumilus* xylosidase (*XLO*1; La Grange et al. 1997), and the *A. niger* α-L-arabinofuranosidase gene (*ABF*2; Crous et al. 1996).

The hope is that these efforts will lead to the development of effective pectolytic, glucanolytic, and xylanolytic wine yeasts that would contribute to the clarification of wine and replace or reduce the levels of commercial enzyme preparations that are needed (Pretorius 2000). Polysaccharide-degrading enzymes secreted by wine yeasts may also improve liquefaction of the grapes, thereby increasing the juice yield and the release of color and flavor compounds entrapped in the grape skins (Dequin 2001; Pretorius and Van der Westhuizen 1991; Van Rensburg and Pretorius 2000).

3.3 Controlled Cell Flocculation and Flotation

Saccharomyces cerevisiae cells can sense a wide range of physical and chemical signals and the yeast adapts its growth pattern in response. These adaptations include yeast filamentation, agglomeration, flocculation, and flotation, which are influenced by a variety of genetic, physiological, and biochemical factors that are not always clearly understood.

Filamentous growth and the formation of pseudohyphae and hyphallike structures often result in dimorphism, which is known to be affected by nutrient limitation and the availability of oxygen (Gagiano et al. 2002). Agglomeration involves an extensive, nonreversible cell aggregation process; flocculation refers to an asexual cellular aggregation when yeast cells adhere to one another reversibly to form microscopic flocs, which sediment out of suspension. Yeast cell flotation, the opposite of flocculation, is the ability of nonaggregated yeast cells to trap CO_2 bubbles in a fermenting liquid and to form a film or vellum at the top of fermentation vessels.

Flocculation in *S. cerevisiae* is mediated by specific calcium-activated lectins, the *FLO* gene-encoded flocculins, which are surface glycoproteins capable of directly binding the mannoproteins of adjacent cells (Teunissen and Steensma 1995). A number of dominant, semidominant, and recessive genes are known to be involved in flocculation. Distinct flocculation phenotypes, designated Flo and NewFlo, have been identified based on their sensitivities to sugar inhibition and proteolytic enzymes (Gagiano et al. 2002). These phenotypes also display different sensitivities to yeast growth conditions, most notably temperature, acidity of the culture medium, and glucose availability (Soares and Mota 1996; Stratford 1996). The flocculation genes include *FLO*1, *FLO*2, *flo*3, *FLO*4, *FLO*5, *flo*6, *flo*7, *FLO*9, *FLO*10, and *FLO*11/*MUC*1 (Lambrechts et al. 1996; Lo and Dranginis 1996; Teunissen and Steensma 1995). The *FLO*11/*MUC*1 gene was also shown to be involved in pseudohyphal development and invasive growth (Lambrechts et al. 1996), while *FLO*8 was reported to encode a transcriptional activator of *FLO*1 and *FLO*11/*MUC*1 (Gagiano et al. 2002). However, in a study of the expression of *FLO*1

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and *FLO*11/*MUC*1 in 25 commercial wine yeast strains, it was found that they are not coregulated (Carstens et al. 1998). It also is unclear what the advantage would be to the yeast cell of coregulating the expression of *FLO*11/*MUC*1 and three glucoamylase-encoding genes (*STA*1, *STA*2, and *STA*3) involved in starch metabolism (Gagiano et al. 2002; Vivier et al. 1997). In fact, the unusually long promoter sequences of *FLO*11/*MUC*1 and *STA*1–3 are almost identical, and it was shown that several transcriptional activators (e.g., Flo8p, Msn1p, and Mss11p) coregulate *FLO*11/*MUC*1-mediated filamentous growth and *STA*1–*STA*3-facilitated starch assimilation (Gagiano et al. 2002; Lambrechts et al. 1996; Webber et al. 1997).

Yeast needs to perform conflicting roles and the regulated expression of the flocculation genes therefore is important in wine production; a high suspended yeast count ensures a rapid fermentation rate during the fermentation of grape must, while efficient settling is needed to minimize problems with wine clarification at the end of sugar conversion (Henschke 1997). Yeast flocculation is especially important for the production of Champagne and bottle-fermented sparkling wine. This traditional method entails the addition of the *liquer de tirage* (sugar and yeast solution) to the *cuvée* (base wine blend) and a secondary fermentation in the bottle (the so-called *prise de mousse*) to develop specific organoleptic characteristics (e.g., a distinct autolysis-mediated fermentation bouquet and mannoprotein-coated bubbles that add to the finesse of the sparkling wine). After about a year, the yeast cells are removed from the bottle by the riddling (*rumage*) and disgorging procedure, which is laborious, time consuming, and expensive (Ribéreau-Gayon et al. 2000). The controlled onset of yeast flocculation at the appropriate time during sparkling wine production can simplify this costly process (Dequin 2001). The native promoter of the *FLO*1 gene therefore was replaced with the *HSP*30 gene promoter, which is known to function during the stress conditions (e.g., nutrient depletion and the presence of high levels of alcohol) that usually prevail during the late stationary phase (Verstrepen et al. 2001). It was shown that the expression of *FLO*1, linked to the late-fermentation *HSP*30 promoter, can be induced by a heat-shock treatment, confirming that controlled flocculation is indeed possible during fermentation (Verstrepen et al. 2001).

Unlike sparkling wine, which requires effective flocculation at the end of the secondary fermentation, flor sherry requires yeast flotation at the end of fermentation. Flor sherry is produced using certain strains of *S. cerevisiae* (formerly known as *S. beticus* and *S. capensis*) that are capable of forming a yeast film on the surface (flor) of a base wine exposed to air. These strains are known for their high ethanol tolerance, superior film-forming ability and desirable oxidative metabolism (Henschke 1997). Initial reports indicated that the vellum-forming trait segregated according to Mendelian rules in the asci of sherry yeasts, yet it now seems unlikely that the flor trait is controlled by a single dominant gene. A number of genes encoding cell wall-associated, hydrophobic proteins have been implicated in vellum formation. Very few yeasts capable of growth on wine are suitable for flor sherry production, and the genotype of sherry yeasts therefore is likely to be more complex than originally expected. However, the relevant genetic and metabolic mechanisms that would allow for controlled vellum formation in flor sherry production may be brought to light once the most important genes responsible for film formation and the characteristic nutty bouquet have been identified. There are, however, unconfirmed, anecdotal indications that the *FLO*11/*MUC*1 gene is involved in biofilm formation that relates to flor formation during sherry aging. This gene seems to be a good candidate to investigate for the possibility of developing sherry yeasts in which vellum formation can be controlled.

4 THE DEVELOPMENT OF YEASTS FOR THE IMPROVEMENT OF THE BIOLOGICAL CONTROL OF WINE SPOILAGE

If there is uncontrolled microbial growth before, during or after wine fermentation, the chemical composition of the end product can be altered, detracting from its sensory properties of appearance, aroma and flavor, as well as from its wholesomeness. If microbial spoilage is severe, the wine becomes unpalatable (Du Toit and Pretorius 2000; Sponholz 1993). As a result of the high initial sugar content, low pH, anaerobic fermentation conditions and high alcohol levels at the end of fermentation, only a few spoilage yeasts and bacteria can survive the strong selective pressures in fermenting grape must and in wine (Henschke 1997).

Spoilage yeasts include species of *Brettanomyces* (the nonsexual, nonsporulating form of *Dekkera)*, the osmotolerant yeast *Zygosaccharomyces* and the film-forming yeast species *Pichia* and *Candida*. *Brettanomyces intermedius* (*Dekkera intermedia*) produces haze, turbidity, volatile acidity, and a mousy taint; *Zygosaccharomyces baillii* causes turbidity after refermentation during the storage of wine or after bottling, resulting in the formation of sediment and a reduction in acidity. Wines spoiled by *Pichia membranaefaciens* and *Candida krusei* taste oxidized and are less acidic (Sponholz 1993).

Although the degree of wine spoilage that can be caused by yeasts must not be underestimated, it is accepted widely that bacteria are the primary culprits, especially acetic acid and lactic acid bacteria. A vinegary taint in wine is often associated with the activity of acetic acid bacteria, such as *Acetobacter aceti*, *Acetobacter pasteurianus*, and *Gluconobacter oxydans* (Du Toit and Pretorius 2002; Sponholz 1993). Although some lactic acid bacteria play an important role in the malolactic fermentation of wine, others may cause serious faults. Excessive volatile acidity, mannitol taint, ropiness, mousiness, acrolein formation and bitterness, tartaric acid degradation, diacetyl overproduction, and rancidness, as well as the very unpleasant geranium off-flavor, are often the result of uncontrolled growth of some species of *Lactobacillus*

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(e.g., *L. brevis*, *L. hilgardii*, *L. plantarum*), *Leuconostoc* (e.g., *L. mesenteroides*), *Streptococcus* (*S. mucilaginosus*) and *Pediococcus* (e.g., *P. cerevisiae*) (Du Toit and Pretorius 2000; Sponholz 1993).

Healthy grapes, cellar hygiene, and sound enological practices (e.g., appropriate pH, fermentation temperature, filtration, application of fining agents, etc.) will remain the cornerstones of the winemaker's strategy against the uncontrolled proliferation of spoilage microbes (Du Toit and Pretorius 2000). Appropriate inoculation levels of *S. cerevisiae* and *Oenococcus oeni* starter cultures will outcompete undesirable contaminants, thereby limiting the risk of spoilage (Fugelsang 1997). Added safety is provided by the addition of chemical preservatives, such as sulfur dioxide that control the growth of spoilage microorganisms. Dimethyl dicarbonate (known as DMDC or Velcorin), benzoic acid, fumaric acid, and sorbic acid are also used in some countries to control microbial growth in must and wine (Du Toit and Pretorius 2000). However, excessive use of these chemical preservatives is harmful to the quality of the wine and related fortified and distilled products, and is confronted by mounting consumer resistance. Due to a shift in consumer preference from heavily preserved and processed, to more natural and healthier products a worldwide search for safe, food-grade preservatives of biological origin has emerged (Du Toit and Pretorius 2000). Among the major focuses of these investigations into novel biopreservatives are the identification and application of effective antimicrobial enzymes (e.g., lysozyme) and peptides (e.g., zymocins and bacteriocins). These efforts have been encouraged by the successful application of lysozyme and nisin to protect beer, wine, and fruit brandies from spoilage lactic acid bacteria and the recent approval by the Office International de la Vigne et du Vin (OIV) of the use of commercial lysozyme preparations to control malolactic fermentation and to stabilize wine afterwards (Du Toit and Pretorius 2000; Ogden et al. 1988). However, wine is a market-sensitive commodity and large-scale industrial application of purified antibacterial enzymes and bacteriocins is expensive, leading to higher retail costs, as in the case of beer production (Ogden et al. 1988). One way to overcome this is by developing wine yeast starter culture strains that produce appropriate levels of efficient antimicrobial enzymes and peptides.

4.1 Wine Yeasts Producing Antimicrobial Enzymes

Antimicrobial enzymes are ubiquitous in nature and fulfil a pivotal function in the defense mechanisms of host organisms against infection by fungi and bacteria (Fuglsang et al. 1995). Hydrolytic antimicrobial enzymes, such as chitinases, β-glucanases and lysozyme, act by degrading key structural components of the cell walls of molds and bacteria. Chitinases and β-glucanases synergistically attack chitin and β-1,3-glucan, the main components of fungal cell walls (Carstens et al. 2003). Lysozyme, an *N*-acetylhexosaminidase, lyses the cell walls of Gram-positive species of bacteria that lack an outer membrane by hydrolyzing the β-1,4-glucosidic linkages of peptidoglycan in the cell wall. The alkaline nature of lysozyme contributes to its antibacterial activity. In addition, Gram-negative bacteria that contain an outer membrane are more sensitive to lysozyme in combination with a chelating agent, such as EDTA, or when lysozyme is modified by perillaldehyde (Fuglsang et al. 1995). Conjugation to galactomanan increases the potency of lysozyme towards Gram-negative bacteria by enabling diffusion of the enzyme across the outer membrane of the target cell (Fuglsang et al. 1995).

The lysozyme-encoding gene from chicken egg white was successfully expressed in *Escherichia coli* and *S. cerevisiae*. The bactericidal action of the recombinant lysozyme against Gram-negative bacteria was enhanced when a pentapeptide was inserted into the C-terminus of *E. coli* (Ibrahim et al. 1992). Research is being done to express a modified lysozyme gene in wine yeast that would avoid hyperglycosylation and broaden its activity to effectively eliminate spoilage by lactic and acetic acid bacteria.

Unlike the direct effect of the antifungal yeast *CTS*1-encoded chitinase and *EXG*1-encoded endoglucanase (Carstens et al. 2003) and the antibacterial lysozyme-encoding gene from chicken egg white, some enzymes have an indirect effect on spoilage microbes. For example, the expression of the *A. niger* glucose oxidase gene cassette (*GOX*1) in *S. cerevisiae* resulted in the production of hydrogen peroxide. This, in turn, led to hyperbaric oxygen toxicity, a result of the peroxidation of bacterial membrane lipids, and a strong oxidizing effect on the bacterial cells, which is the cause of the destruction of basic molecular structures, such as nucleic acids and cell proteins (Malherbe et al. 2003). Consideration is currently also been given to the late expression of a catalase gene in wine yeast, which can eliminate excessive *GOX*1-generated hydrogen peroxide after it has played a preservative role during the earlier phases of winemaking.

4.2 Wine Yeasts Producing Antimicrobial Peptides

4.2.1 Zymocins

Over the years, several attempts have been made to expand the zymocidal activity of *S. cerevisiae* so that it could also eliminate other yeast contaminants. Different killer types of *S. cerevisiae* have been hybridized by mating, cytoduction, and spheroplast fusion and, in one case, a DNA copy of the K_1 dsRNA was integrated into the genome of a K_2 strain of *S. cerevisiae* (Boone et al. 1990). However, even a K_1/K_2 double killer *S. cerevisiae* can inhibit only a limited variety of yeast contaminants during wine fermentations. Instead, attention is now focused on the identification of genes encoding more effective zymocins in other yeasts, such as *Pichia* and *Hanseniaspora*, and their possible introduction into *S. cerevisiae*.

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4.2.2 Bacteriocins

Bacteriocin production is a characteristic typical of many lactic acid bacteria. Bacteriocins of lactic acid bacteria are ribosomally synthesized and inhibit closely related bacteria by destabilizing the function of the cytoplasmic membrane. The bacteriocin-producing strain resists its own bacteriocin by producing a highly specific immunity factor (Du Toit and Pretorius 2000). Bacteriocins are divided into three classes, based on their primary structure, molecular mass and heat stability: (Class I) lantibiotics, which are small, heat stable and contain lanthionine, e.g., nisin; (Class II) nonlantibiotics, which are small and heat stable, e.g., pediocin PA-1, leucocin B-Ta11a; and (Class III) large and heat labile, e.g., helveticin J (Du Toit and Pretorius 2000). The bacteriocins of lactic acid bacteria have received considerable attention due to their potential application as natural preservatives. They may provide a valuable, additional, and controllable tool for the inhibition of some deleterious wine-associated organisms.

In a recent study, the bacteriocins nisin, pediocin, and leucocin were evaluated for use as biopreservatives in wine, as a potential alternative to sulfur dioxide (Du Toit 2003). Several type, reference and wine-isolated strains of lactic acid bacteria were found to be sensitive, in varying degrees, to the three bacteriocins, but no sensitivity was detected for wine-associated acetic acid bacteria and yeasts. Pediocin appeared to be stable for a sufficient time in a simulated wine environment. Various combinations of nisin, pediocin, and leucocin were tested against the wine isolate, *Leuconostoc mesenteroides* DIIIM:I, where although the nisin-leucocin was found to be effective, a combination of all three bacteriocins proved to be the most effective. The effect of bacteriocins on the cell morphology of the lactic acid bacteria was examined by scanning electron microscopy and major cell disturbances were detected, especially at the septa where the cells are joined. Using a microtiter broth dilution method, both synergistic and antagonistic activities were observed between certain pairs of bacteriocins. The efficacy, however, depended on the ratio of bacteriocins used in combination (Du Toit 2003).

Although bacteriocins provide the winemaking community with a safe alternative to chemical preservation, their low cost efficiency is a limiting factor. In an attempt to neutralize the cost factor, the feasibility has been investigated of controlling spoilage bacteria during wine fermentations by engineering bactericidal strains of *S. cerevisiae*. Two bacteriocin operons, the *Pediococcus acidilactici PED*1 gene encoding a pediocin and the *Leuconostoc carnosum LCA*1 gene encoding a leucocin, have been successfully expressed in *S. cerevisiae* under control of the *ADH*1 gene promoter and terminator sequences (Schoeman et al. 1999). Secretion of the yeast *PED*1-encoded pediocin and the *LCA*1-encoded leucocin was directed by the *MF* α1-encoded leader peptide. The pediocin operon of *P. acidilactici* consists of four clustered genes, namely *pedA* (encoding a 62 amino acid precursor of the PA-1 pediocin), *pedB* (encoding an immunity factor), *pedC* (encoding a PA-1 transport protein) and *pedD* (encoding a protein involved in the transport and processing of PA-1) (Du Toit 2003). Two genes make up the leucocin operon of *L. carnosum*: *lcaB* (encoding a 61 amino acid precursor of the B-Ta11a leucocin) and $lcaB_1$ (encoding a 113 amino acid immunity factor (Felix et al. 1994).

Biologically active antimicrobial peptides produced by the *S. cerevisiae* transformants were indicated to be present by agar diffusion assays against sensitive indicator bacteria, such as the well-known food pathogen, *Listeria monocytogenes*, and the ferocious wine spoilage bacterium, *Lactobacillus kunkeei*. The heterologous peptides were present at relatively low levels in the yeast supernatant, but pediocin and leucocin activities were detected readily when intact yeast colonies were used in sensitive strain overlays. These promising, preliminary results encouraged further investigations, which are focused on the increase in the extracellular levels of pediocin and leucocin in *S. cerevisiae* and the coexpression of *PED*1 and *LCA*1 together with genes encoding other bacteriocins and bacteriolytic enzymes. This approach will broaden the spectrum of unwanted bacteria that would be inhibited by an engineered wine yeast strain, thereby paving the way for the production of wine with reduced levels of potentially harmful chemical preservatives.

5 THE DEVELOPMENT OF YEASTS FOR THE IMPROVEMENT OF WINE WHOLESOMENESS

Until the 18th century, wine played an integral role in medical practice. Not only was it safer to drink than most available water, but also its alcohol, antioxidant, and acid content inhibited the growth of many spoilage and pathogenic organisms. The paradigm shifted towards the second half of the 20th century, when alcohol consumption, including wine drinking, became the target of health campaigners, who, with some success, demanded warning labels on wine bottles. However, substantial medical evidence accumulated during the 1990s pointed out that the moderate consumption of red wine in particular could reduce the incidence of coronary heart disease (Armstrong et al. 2001).

The principal protective compounds found in wine include the phenolic compounds, resveratrol, salicylic acid and alcohol (Armstrong et al. 2001). Interest in these compounds has been spurred by the dietary anomaly commonly referred to as the "French paradox." This phenomenon refers to a remarkable association between a high fat diet and a lower incidence of coronary heart disease found in Mediterranean countries, in contrast to a higher incidence of coronary heart disease among most other Western cultures. Several possible explanations have been offered, but the best case for resolving this paradox has been made for red wine phenolics that chemically modify blood lipoproteins in cholesterol-furred arteries. The increased benefits of red wine over white wine arise from the processes used to produce the wines. During red wine vinification, the grape must is fermented with the

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grape skin. As the yeast converts the grape sugars to ethanol and carbon dioxide, the fermentation temperature and alcohol content increase. This increases the solubility of the various compounds found in the grape skin, i.e., phenolic acids, anthocyanins, tannins, resveratrol etc., and they are extracted into the wine. During white wine vinification, the grape juice generally is first separated from the skins before it is fermented in order to obtain a clear wine that is low in tannins and other phenolics.

Today, it is generally accepted that moderate wine drinking can be socially beneficial and can be effective in the management of stress and the reduction of coronary heart disease. Prudent wine drinkers, however, continue to monitor their drinking habits to ensure that the benefits exceed the risks. In this regard, they are increasingly fastidious about the presence of undesirable compounds in wine. These unwanted compounds include suspected carcinogens, such as ethyl carbamate, neurotoxins, such as biogenic amines, and asthmatic chemical preservatives, such as sulfites (Pretorius 2000). The most finicky among these fussy wine drinkers are even concerned about high levels of alcohol in wine. When wine yeast strains are developed, it therefore is of the utmost importance to focus on these health aspects and to develop yeasts that may enhance the benefits and reduce the risks associated with moderate wine consumption.

5.1 Increased Production of Resveratrol

Phytoalexins, including stilbenes such as resveratrol, are known to reduce the risk of coronary heart disease. Resveratrol acts as an antioxidant and antimutagen, thereby displaying antiinflammatory and cancer chemopreventive activity, as well as the ability to induce specific enzymes that metabolize carcinogenic substances.

Stilbenes are secondary plant products produced through the phenylalanine/polymalonate pathway. Resveratrol is a stress metabolite that is produced by grapes during fungal infection, wounding or ultraviolet radiation. The actual amount of resveratrol found in wine varies considerably and is dependent on a number of factors, including climate, cultivar, fungal pressure in the vineyard, and the influence of enological procedures. Resveratrol is synthesized mainly in the skin cells of grape berries and only traces are found in the flesh. As a result, red wine has a much higher resveratrol concentration than white wine due to skin contact during the first phase of fermentation.

Developing wine yeasts that can produce resveratrol during fermentation can increase the levels of resveratrol in both red and white wine. To achieve this goal, the phenylpropanoid pathway in *S. cerevisiae* will have to be modified to produce ρ-coumaroyl-coenzyme A, one of the substances for resveratrol synthesis. The other substrate, malonyl-coenzyme A, is already found in yeast and is involved in *de novo* fatty acid biosynthesis. It is hypothesized that the production of ρ-coumaroyl-coenzyme A can be achieved by introducing the phenylalanine ammonia-lyase gene (*PAL*), the cinnamate 4-hydroxylase gene (*D4H*), the coenzyme A ligase gene (4*CL*9 and 4*CL*216) and the grape stilbene synthase gene (*Vst*1) in *S. cerevisiae*. The introduction of the grape stilbene synthase gene (*Vst*1) may then catalyze the addition of three acetate units from malonyl-coenzyme A to *p*-coumaryl-coenzyme A, resulting in the formation of resveratrol. In a preliminary study, the *4CL9,* 4*CL*216 and *Vst*1 genes were cloned under the control of strong yeast promoters of the alcohol dehydrogenase II (*ADH*2) and enolase (*ENO*2) genes and transformed into *S. cerevisiae* (Becker 2002. Initially, no resveratrol could be detected in the yeast expressing the coenzyme A ligase and resveratrol synthase genes. Only after the samples were treated with β-glucosidase to release the glucose moieties from the so-called piceid molecule (the glucoside form of resveratrol) were detectable levels perceived in mass spectrometric assays. This has encouraged efforts to coexpress the coenzyme A ligase and resveratrol synthase genes, together with a glucose-resistant β-glucosidase gene, such as the *BGL*1 gene from *S. fibuligera*. However, this must be investigated in greater depth before it can be recommended as a way to develop resveratrol-producing wine yeast strains.

5.2 Reduced Formation of Ethyl Carbamate

Ethyl carbamate (also known as urethane) is a suspected carcinogen found in most fermented foods and beverages. The potential health hazard has led to a growing demand from consumers and liquor-control authorities to reduce the permitted levels of ethyl carbamate in wines and related products. Young wines do not contain measurable levels of ethyl carbamate, but the required precursors are present, and these can generate a considerable amount of this mutagenic compound when wine is aged or stored at elevated temperatures. Beverages with high levels of alcohol, such as sherry, port, dessert wines, and distilled products, also tend to contain much higher levels of ethyl carbamate than table wine (Pretorius 2000). It is thought that ethyl carbamate is formed in aging wines, fortified wines, and brandies from the reaction between urea and ethanol. Consequently, excessive application of urea-containing fertilizers to vines and spraying of urea shortly before harvest to remove leaves are not recommended. In addition, the use of urea-containing nutrient supplements for yeast to avoid stuck or sluggish fermentations is prohibited during wine fermentations. Besides these factors that could lead to high urea levels and concomitant transgression of ethyl carbamate limits, *S. cerevisiae* strains also vary widely with regard to their ability to form urea (Ough et al. 1991).

In *S. cerevisiae*, urea is formed by the *CAR*1-encoded arginase during the breakdown of arginine, one of the main amino acids in grape juice. In this process, arginine is converted to ornithine, ammonia, and carbon dioxide, and urea is formed as an intermediate product. Urea is secreted into the wine by certain yeast strains that depending on fermentation conditions, may be unable to further metabolize

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the external urea. Even though all *S. cerevisiae* strains secrete urea, the extent to which they reabsorb the urea differs (An and Ough 1993). More urea is secreted by *S. cerevisiae* at higher fermentation temperatures, whereas high ammonia concentrations suppress the reabsorption of urea by the yeast. This means that it is important to inoculate grape must with a wine yeast strain that produces little urea when the juice has a high arginine content.

Strain selection is one of the ways to reduce the accumulation of urea in wine. As another means of curbing ethyl carbamate formation in the end product, the successive disruption of the *CAR*1 arginase gene in an industrial saké yeast successfully eliminated urea accumulation in rice wine (Kitamoto et al. 1991). This arginase deletion mutation led to a yeast strain that could not metabolize arginine. However, growth was also impeded that limits the commercial use of the strain.

Another possibility is to add commercial preparations of acidic urease that enables the hydrolysis of urea in wine (Ough and Trioli 1988). This practice was recently approved by the OIV and is used in some wine-producing countries to lower ethyl carbamate levels in wines and related products. A cheaper route to lower levels of ethyl carbamate would be to develop a wine yeast strain that produces an extracellular, acidic urease. In one such attempt, a novel urease gene was constructed by fusing the α, β, and γ subunits of the *Lactobacillus fermentum* urease operon (Visser 1999). Jack bean urease linker sequences were also inserted between the α and β, as well as the β and γ subunits. These two gene constructs were successfully expressed under the control of the *S. cerevisiae PGK*1 promoter and terminator signals in the yeasts *S. cerevisiae* and *S. pombe*. The level of transcription in *S. cerevisiae* was much higher than in *S. pombe*, but the secretion of urease peptides was extremely low (Visser 1999). In contrast to the *S. pombe* urease, the *S. cerevisiae*-derived urease was unable to convert urea into ammonia and carbon dioxide. The fact that there is no recombinant urease activity in transformed *S. cerevisiae* cells is probably the result of a lack of the essential auxiliary proteins, which are present only in urease-producing species such as *S. pombe*. Without these proteins, *S. cerevisiae* is unable to assemble the various subunits into an active urease. It seems, therefore, that for *S. cerevisiae* to be able to express an active urease, accessory genes of *L. fermentum* will also have to be cloned and expressed in addition to the structural urease genes.

5.3 Reduced Formation of Biogenic Amines

Biogenic amines are organic, basic compounds that occur in various foods and beverages, including fermented products such as beer and wine. These compounds originate mainly from the decarboxylation of precursor amino acids by substrate specific enzymes of the microorganisms present in the food or beverage. Histidine, tyrosine, phenylalanine, and lysine are the precursors of histamine, tyramine, phenethylamine, and cadaverine respectively, whereas polyamines, such as putrescine, spermine, and spermidine, are synthesized from ornithine and arginine (Torrea Goñi and Ancín Azpilicueta 2001). The ingestion of products containing relatively high levels of biogenic amines can provoke adverse effects, such as hypotension, flushing, headache, and migraine. Putrescine is the amine that is generally present at the highest concentration in wine, and it is known as the most effective potentiator of histamine toxicity in humans (Guerrini et al. 2002). Moreover, putrescine is one of the biogenic amines that are potential precursors of carcinogenic nitrosamines. The presence of biogenic amines in wine therefore is increasingly becoming a health concern for the consumer.

A recent investigation of the influence of different strains of *S. cerevisiae* on the concentration of biogenic amines in wine revealed that only slight differences in the content of the amines could be detected for the various strains tested, and high concentrations were never reached (Torrea Goñi and Ancín Azpilicueta 2001). Moreover, no relationship was found between the concentration of biogenic amines in wine and the utilization of their precursor amino acids by the different strains of *S. cerevisiae* during the alcoholic fermentation. This confirmed the notion that biogenic amines in wine are produced mainly by spoilage bacteria (e.g., *Lactobacillus*, *Leuconostoc*, and *Pediococcus*), as well as by the malolactic fermentation bacteria (e.g., *Oenococcus*) (Guerrini et al. 2002). Although it has been reported that several strains of these lactic acid bacteria (including some strains of *O. oeni*) are responsible for the accumulation of biogenic amines in wine (red wine usually being richer in amines than white wine), *Pediococcus* is the main culprit for the accumulation of histamine and other biogenic amines that exceed the thresholds for sensitive wine drinkers (Guerrini et al. 2002). Higher doses of sulfur dioxide to combat *Pediococcus* and other bioamine-producing lactic acid bacteria are also not the answer to this problem, because consumer resistance to the presence of asthmatic chemical preservatives is mounting.

A logical approach to reduce the levels of biogenic amines in wine seems to be the development of bactericidal wine yeast starter culture strains that would curb the growth of lactic acid bacteria in wine. As discussed in Section 4, the construction of wine strains of *S. cerevisiae* that secrete a wide spectrum of bacteriolytic enzymes (e.g., lysozyme) and bacteriocins (e.g., pediocin and leucocin) would undoubtedly contribute to the inhibition of bioamine-producing lactic acid bacteria in wine (Du Toit 2003; Du Toit and Pretorius 2000; Schoeman et al. 1999). Furthermore, the development of a malolactic wine yeast strain has also been proposed as an alternative to the use of lactic acid bacteria, such as *O. oeni*, to conduct the malolactic fermentation (see Section 6.5), thereby avoiding the risk of bacterially produced biogenic amines in wine (Volschenk et al. 1997).

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5.4 Decreased Levels of Alcohol

The effects of alcohol consumption on cardiovascular disease are complex and remain under close scrutiny. While heavy alcohol intake increases overall mortality and mortality due to cardiovascular complications, the moderate intake of alcohol appears to exhibit a protective effect against coronary heart disease when compared to drinking no alcohol at all (Armstrong et al. 2001). The mechanisms by which alcohol exerts its protective effect are not completely understood. One of the best established physiological risk factors for vascular disease, i.e., decreased serum levels of high-density lipoprotein, has been shown to increase as a direct result of moderate alcohol consumption. High-density lipoproteins are particles that are able to scavenge cholesterol from membranes and tissues where it is in excess and transport it to the liver for excretion. Alcohol is able to increase, in a dose-dependent manner, the hepatic secretion of apolipoprotein A-I and apolipoprotein A-II that are the primary lipoproteins found in the high-density lipoproteins. Alcohol is also able to block the stimulation of phospholipase A_2, thereby preventing the mobilization of arachidonate, which is essential for the generation of thromboxin A_2, an important platelet aggregant. However, the amount of alcohol required for this mechanism of protection far exceeds what can be described as moderate consumption (Armstrong et al. 2001).

Apart from the beneficial health issues related to moderate alcohol consumption as opposed to the excessive intake of alcohol, there are several reasons why consumers shy away from beverages containing high levels of alcohol. Health and fitness consciousness and the strict traffic laws pertaining to "drinking and driving" seem to be the main reasons for the worldwide decline in the consumption of alcohol. Thanks to this trend in consumer behavior, there is an increasing demand for low alcohol, reduced alcohol, and dealcoholized wines. This has put a great deal of pressure on wine producers, particularly those in the warm climate wine-producing regions, where grape sugar levels are much higher than in cooler climates. Moreover, there is a tendency nowadays to leave the grapes on the vines for longer in order to ensure ripeness not only in terms of sweetness, but also with respect to the ripeness and "softness" of the potentially astringent phenolics in wine. This tendency has led to even higher levels of sugar in grapes and ethanol in wine. Such wines are prone to alcohol levels of 13.5–15% alcohol or even higher. An additional incentive for the production of wines with decreased levels of alcohol has to do with the producers' interest in the potential for savings in taxes and tariffs (Gladstones 2000).

Several physical and chemical processes (sometimes used in combination) have been developed for the partial removal of alcohol from wine, including thermal evaporation, distillation, membrane filtration, adsorption, centrifugation, freeze concentration, and partial fermentation (Pickering et al. 1999a–c). The use of reverse osmosis and the so-called spinning cone method are probably the most commonly used methods to decrease the alcohol content of wine. The negative views of some traditionalists about these intensive processing procedures, together with the high costs involved, have encouraged the search for cheaper and less "artificial" biological methods to adjust the levels of alcohol in wine.

The treatment of grape must with glucose oxidase has recently received a lot of interest as a biological alternative to the above-mentioned physical and chemical processes Pickering et al. 1999a–c). When grape must is treated with the *A. niger* glucose oxidase, some of the glucose is converted to gluconic acid, thereby resulting in wine with decreased levels of alcohol and a slightly lower pH. As an extension of this enzymatic approach to the production of low alcohol wine, the *A. niger GOX*1 gene was expressed in *S. cerevisiae* under control of the *PGK*1 promoter (Malherbe et al. 2003). In microvinification trials, the alcohol content of the untransformed yeast was 1.8–2% lower and the titratable acidity was higher than that of the control wine.

Another biological approach to the production of low alcohol wine, which could be used in conjunction with the glucose oxidase-producing yeast strain, entails the overproduction of glycerol at the expense of ethanol. About 4–10% of the carbon source is usually converted to glycerol, resulting in glycerol levels of 7–10% of that of ethanol (Scanes et al. 1998). The amount of glycerol in wines depends on many factors: grape variety, nitrogen composition, degrees of ripeness (sugar levels) and mold infection (during which glycerol is produced), sulfite levels and the pH of grape must, fermentation temperature, aeration, and choice of starter culture strain and inoculation level (Remize et al. 1999; Scanes et al. 1998). Slight increases in glycerol production in wine can be achieved by using yeast strains selected or bred for high glycerol production and by optimizing fermentation conditions. More recently, it was reported that the overexpression of the glycerol-3-phosphate dehydrogenase genes (*GPD*1 and *GPD*2), together with constitutive expression of the glycerol transport facilitator gene (*FPS*1), successfully redirected the carbon flux towards glycerol and the extracellular accumulation of glycerol (see Section 6.4). It remains to be proved whether the heterologous expression of the *A. niger GOX*1 gene, together with the overexpression of the *S. cerevisiae GPD1, GPD*2 and *FPS*1 genes in wine yeast strains (that carry an ald6 deletion allele), would provide a viable means of lowering the alcohol content to appropriate levels in commercial wines.

6 THE DEVELOPMENT OF YEASTS FOR THE IMPROVEMENT OF WINE FLAVOR AND OTHER SENSORY QUALITIES

The single most important factor in winemaking is the organoleptic quality of the final product. The bouquet of a wine is determined by the presence of a well-balanced ratio of desirable flavor compounds and metabolites, and the absence of undesirable ones (Lambrechts and Pretorius 2000).

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A number of variables make a contribution to the distinctive flavor of wine, brandy, and other grape-derived alcoholic beverages. Vine development and berry composition are affected by the grape variety, viticultural practices and *terroir*, which also exert major influences on the distinctiveness of wine and brandy flavor (Lambrechts and Pretorius 2000). Enological practices, including the yeast and fermentation conditions, have a prominent effect on the primary flavors of wines. The components that are formed during fermentation dominate the volatile profile of wines, as these compounds are present in the highest concentrations immediately after fermentation or distillation, the flavor of wine and brandy only approximates that of the finished product (Lambrechts and Pretorius 2000). After the sudden and dramatic changes in composition during fermentation and distillation, chemical constituents generally react slowly during aging to move to their equilibria, resulting in gradual changes in flavor. Subsequently, the harmonious complexity of wine and brandy can be increased further by volatile extraction during oak barrel aging (Lambrechts and Pretorius 2000).

Although a great deal of information has been published on flavor chemistry, odor thresholds and aroma descriptions, the flavor of complex products such as wine and brandy cannot be predicted. With a few exceptions (e.g., terpenes in the aromatic varieties and alkoxypyrazines in the vegetative or herbaceous cultivars), the perceived flavor is the result of specific ratios of many compounds rather than being caused by a single "impact" compound. Esters and alcohols, the major products of yeast fermentation in wines and brandies, contribute to a generic background flavor, whereas subtle combinations of trace components derived from the grapes usually elicit the characteristic aroma notes of these complex beverages.

6.1 Enhanced Liberation of Grape Terpenoids

The varietal flavor of grapes is determined mainly by the accumulation and profile of volatile secondary metabolites in grapes (Henschke 1997). However, a great number of these metabolites occur as their respective, nonvolatile *O*-glycosides. Various studies have shown that increased enzymatic hydrolysis of the aroma precursors present in grape juice can liberate the aglycon to intensify the varietal character of wines (Canal-Llauberes 1993). For example, terpenols such as geraniol and nerol can be released from terpenyl-glycosides by the grape-derived β-D-glycosidase activity present in muscat grape juice. However, grape glycosidases are unable to hydrolyze sugar conjugates of tertiary alcohols such as linalool (Canal-Llauberes 1993). In addition, these grape enzyme activities are inhibited by glucose and exhibit poor stability at the low pH and high ethanol levels of wine (Henschke 1997). As a result of these limiting characteristics of grape-derived glycosidases and the fact that some of the processing steps during the clarification of must and wine profoundly reduce their activity, these endogenous enzymes of grapes have only a small effect in enhancing varietal aroma during winemaking (Canal-Llauberes 1993).

Aroma-liberating β-glucosidases from *Aspergillus* and other fungal species have been developed as commercial enzyme preparations to be added to fermented juice (as soon as the glucose has been consumed by the yeast) or to young wine (Canal-Llauberes 1993) as an alternative to the inefficient grape glycosidases. However, it is expensive to add exogenous enzyme preparations to wine, and the process is viewed by many traditionalists as an "artificial" or "unnatural" intervention by the winemaker. This has led to renewed interest in the more active β-glucosidases produced by certain strains of *S. cerevisiae* and other wine-associated yeasts (Restuccia et al. 2002).

In contrast to the grape β-glycosidases, yeast β-glucosidases are not inhibited by glucose, and the liberation of terpenols during fermentation can be ascribed to their action on the terpenyl-glycoside precursors (Canal-Llauberes 1993). These β-glucosidases are absent from most *S. cerevisiae* starter culture strains and the β-glucosidase gene (*BGL*1) of the yeast *S. fibuligera* therefore was expressed in *S. cerevisiae*. The aroma intensity of wine increased when the β-1,4-glucanase gene from *Trichoderma longibrachiatum* was expressed in wine yeast, presumably due to the hydrolysis of glycosylated flavor precursors (Pérez-González et al. 1993). Likewise, the *S. cerevisiae* exo-β-1,3-glucanase gene (*EXG*1) was overexpressed and the endo-β-1,4-glucanase gene (*END*1) from *B. fibrisolvens*, the endo-β-1,3-1,4-glucanase (*BEG*1) from *B. subtilis* and the α-arabinofuranosidase (*ABF*2) (Van Rensburg and Pretorius 2000) were introduced in *S. cerevisiae*. Trials are underway to determine the effect of these transgenic yeasts on the varietal character of muscat wines.

6.2 Enhanced Production of Desirable Volatile Esters

During alcoholic fermentation, wine yeast produces ethanol, carbon dioxide, and a number of byproducts, including esters, of which alcohol acetates and C_4–C_{10} fatty acid ethyl esters are found in the highest concentration in wine and brandy (Nykänen 1986). Although these compounds are present in all wines, the levels of esters formed vary significantly. The ester concentration produced during fermentation is not only dependent on factors such as grape cultivar, rootstock, and grape maturity, but also on the yeast strain, fermentation temperature, insoluble material in the grape must, vinification methods, skin contact, must pH, the amount of sulfur dioxide, amino acids present in the must, and malolactic fermentation (Cole and Noble 1995). The ester content of distilled beverages is also greatly dependent on whether the yeast lees is present during distillation (Lambrechts and Pretorius 2000; Nykänen 1986).

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The characteristic fruity odors of wine are caused mainly by a mixture of hexyl acetate, ethyl caproate and caprylate (applelike aroma), iso-amyl acetate (bananalike aroma), and 2-phenylethyl acetate (fruity, flowery flavor with a honey note). At least three acetyltransferase activities are responsible for the synthesis of acetate esters, such as iso-amyl acetate and ethyl acetate, in *S. cerevisiae*: alcohol acetyltransferase (AAT), ethanol acetyltransferase (EAT) and iso-amyl alcohol acetyltransferase (IAT) (Malcorps et al. 1991). These acetyltransferases are sulfhydryl (SH) enzymes that react with acetyl coenzyme A (acetyl-CoA) and, depending on the degree of affinity, with various higher alcohols to produce esters (Fujii et al. 1997). These enzymatic activities are also known to be repressed strongly under aerobic conditions and by the addition of unsaturated fatty acids to a culture.

Of all the acetyltransferases, the *ATF*1-encoded AAT activity has been studied best in *S. cerevisiae*. Atf1p is located within the yeast's cellular vacuomes and plays a central role in the production of iso-amyl acetate and, to a lesser extent, ethyl acetate during beer fermentation. When the *ATF*1 gene was overexpressed under the control of the *PGK*1 promoter, the transformed wine yeast strains produced wine and distillates with significantly elevated levels of ethyl acetate, iso-amyl acetate and 2-phenylethyl acetate (Lilly et al. 2000). There were only minor changes in the concentration of ethyl caprate, ethyl caprylate and hexyl acetate, whereas the acetic acid concentration decreased by more than half. These changes in the wine and distillate composition had a marked effect on the solvent/chemical (associated with ethyl acetate and iso-amyl acetate), herbaceous and heads-associated aroma of the final distillate and the solvent/chemical and fruity/flowery character of Chenin blanc wines (Lilly et al. 2000). This study established the concept that the overexpression of acetyltransferase genes, such as *ATF*1, could have a profound effect on the flavor profiles of wines and distillates deficient in aroma, thereby paving the way for the production of products maintaining a fruitier character for longer periods after bottling.

6.3 Optimized Fusel Oil Production

Alcohols with carbon numbers higher than that of ethanol, such as isobutyl, isoamyl, and active amyl alcohol, are called fusel oil. These higher alcohols are produced by wine yeasts during alcoholic fermentation from intermediates in the branched chain amino acids pathway, leading to the production of isoleucine, leucine, and valine by decarboxylation, transamination, and reduction (Webb and Ingraham 1963). When present at high concentrations, these higher alcohols have undesirable sensory characteristics, but in wine they are usually present at concentration levels below their threshold values and they sometimes could even contribute to wine quality (Giudici et al. 1990; Kunkee and Amerine 1970). However, since higher alcohols are concentrated by the distilling process, it is important that they are reduced in wines that are to be distilled for brandy production (Snow 1983).

Initial attempts to use Ile^-, Leu^- and Val^- auxotrophic mutants were successful in lowering the levels of isobutanol, active amyl alcohol, and isoamyl alcohol produced in fermentations. However, because their growth and fermentation rates were compromised, these mutants were of no commercial use (Ingraham et al. 1961). A Leu^- mutant derived from the extensively used Montrachet wine yeast was reported to produce more than 50% less isoamyl alcohol during fermentation than the prototrophic parent (Snow 1983). It will be very interesting to see whether the integrative disruption of specific *ILE*, *LEU*, and *VAL* genes of wine yeasts will result in lower levels of fusel oil in wine for distillation.

6.4 Enhanced Glycerol Production

Glycerol is nonvolatile and therefore does not have a direct impact on the aromatic characteristics of wine. However, this triol imparts other sensory qualities; it has a slightly sweet taste and, because of its viscous nature, it contributes to the smoothness, consistency, overall body, and mouth feel of wine (Scanes et al. 1998). Under controlled conditions, glycerol concentrations typically are higher in red wines than in white wines, varying from 1 to 15 g/l (Scanes et al. 1998). The threshold taste level of glycerol is 5.2 g/l in wine, whereas a change in the viscosity is only perceived at a level of 25 g/l (Scanes et al. 1998). Wine yeast strains that overproduce glycerol therefore could be valuable in improving the organoleptic quality of wine (Michnick et al. 1997; Remize et al. 1999).

The main role of glycerol synthesis during wine fermentations is to supply the yeast cell with a solute that is responsive to osmotic stress and to equilibrate the intracellular redox balance by converting the excess NADH generated during biomass formation to NAD^+ (Scanes et al. 1998). The formation of glycerol involves the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, a reaction catalyzed by the *GPD*1 and *GPD*2-encoded glycerol-3-phosphate dehydrogenase isozymes. This is followed by the dephosphorylation of glycerol-3-phosphate to glycerol by glycerol-3-phosphatase (Remize et al. 1999). The primary role of the *GPD*1-encoded activity is osmoadaptation (Albertyn et al. 1994), whereas the *GPD*2-encoded activity is involved in maintaining the redox balance, particularly during anaerobic growth (Ansell et al. 1997).

Overexpression of either *GPD*1 or *GPD*2 in *S. cerevisiae* produces wines with significantly higher levels of glycerol (De Barros Lopes et al. 2000; Michnick et al. 1997; Remize et al. 1999). A lower biomass concentration was attained in the strains overexpressing *GPD*1, probably due to high acetaldehyde production during the growth phase (Remize et al. 1999). It was interesting that the fermentation rate during the stationary phase of wine fermentation was stimulated in these strains, suggesting that the availability of NAD may control the rate of glycolytic flux (Remize et al.

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1999). Other side-effects of these glycerol-overproducing yeasts included the accumulation of byproducts, such as pyruvate, acetate, acetoin, 2,3-butanediol, and succinate (De Barros Lopes et al. 2000; Eglinton et al. 2002; Michnick et al. 1997; Remize et al. 1999).

Recently, a method was devised to overcome the most negative side effect of glycerol overproduction, namely a marked increase in acetate formation. Since acetaldehyde dehydrogenases were shown to play a prominent role in acetate formation, the *ALD*6 and *ALD*7 genes, encoding a cytosolic Mg^{2+}-activated, NADP-dependent and a mitochondrial K^{+}-activated, NAD(P)-dependent acetaldehyde dehydrogenase respectively, were disrupted. A wine yeast strain in which *GPD*1 or *GPD*2 was overexpressed in conjunction with the deletion of *ALD*6 produced two- to three-fold more glycerol and a similar amount of acetate compared to the untransformed strain (Eglinton et al. 2002; Remize et al. 1999). To maintain the redox balance in these recombinant wine yeasts, the formation of succinate and 2,3-butanediol was increased to concentrations remaining in the range of that found in wine. When the metabolome of these strains was analyzed, it was revealed that the profile of some secondary metabolites of fermentation, including acids, esters, aldehydes, and higher alcohols, many of which are flavor-active in wine, was different to that of the control wine (Eglinton et al. 2002). Thus, modifying the expression of *GPD*1 or *GPD*2, together with *ALD*6, represents an effective strategy to increase the glycerol and decrease the ethanol concentration during fermentation, and alters the chemical composition of wine in such a way that, potentially, novel flavor diversity is possible (Eglinton et al. 2002).

6.5 Bio-adjustment of Wine Acidity

The acidity of grape juice and wine plays an important role in many aspects of winemaking and wine quality, including the sensory quality of the wine and its physical, biochemical and microbial stability. The acidity of the juice and wine, in particular the pH, has a profound influence on the survival and growth of all microorganisms; the effectiveness of antioxidants, antimicrobial compounds and enzyme additions; the solubility of proteins and tartrate salts; the effectiveness of bentonite treatment; the polymerization of color pigments; and the oxidative and browning reactions (Boulton et al. 1996). A large number of organic and inorganic acids are present in wine. The most common organic acids are tartaric and malic acid, which account for 90% of the titratable acidity of grapes. The most important features of wine acidity include the acids themselves, the extent of their dissociation, the titratable acidity and the pH. Among the factors affecting the pH and titratable acidity of grapes are soil potassium and soil moisture; the nature of the rootstock and characteristics of the root system; viticultural practices such as canopy management and irrigation; climatic conditions and prevailing temperature during ripening; the cultivar; and the final berry volume at harvest (Boulton et al. 1996). The climatic conditions and ambient temperature are the factors that have a critical effect on grape maturation and the resulting acidity of the fruit (Ribéreau-Gayon et al. 2000). Under certain climatic conditions, imbalances in the acidity of wines can be caused by the development of acidic compounds in the grape during maturation and the subsequent physical and microbial modification of these compounds during the process of winemaking. If the acidity of such wines with suboptimal pH values is not adjusted, the wines will be regarded as unbalanced or spoilt. In cooler climates, chemical adjustment generally entails a reduction in titratable acidity by physicochemical practices such as blending, chemical neutralization by double salting (addition of calcium carbonate) and precipitation. These processes can reduce wine quality and require extensive labor and capital input.

In warmer viticultural regions blessed with adequate sunshine during the growing season and grape ripening period, malic acid is catabolized at a faster rate. In this case, adjustment of wine acidity generally entails increasing the titratable acidity or, more critically, lowering the pH by adding tartaric acid and sometimes malic and citric acids, depending on the laws of the country. The addition of calcium carbonate and acids are highly contentious practices that sometimes affect free trade in wine, and therefore several laboratories have explored biological alternatives in order to minimize such chemical intervention.

Biodeacidification of wine is currently mediated by lactic acid bacteria, in particular *O. oeni*. Malic acid is decarboxylated to L-lactic acid and carbon dioxide during malolactic fermentation. In addition to reducing the total acidity of wine, malolactic fermentation also enhances microbiological stability and presumably improves the organoleptic quality of wine (Davis et al. 1985). However, nutrient limitation, low temperature, acidic pH, and high alcohol and sulfur dioxide levels often cause the malolactic bacteria to grow poorly in wine, thereby complicating the management of this process. Stuck or sluggish malolactic fermentation often causes spoilage of wine. Several alternatives have been explored, including the possible use of malate-degrading yeasts. Malo-ethanolic fermentations conducted by the fission yeast *Schizosaccharomyces pombe* caused malate to be converted effectively to ethanol, but off-flavors were produced (Volschenk et al. 1997). Attempts to fuse wine yeasts with malate-assimilating yeast were also not successful (Radler 1993). The use of high-density cell suspensions of several yeasts, including *S. cerevisiae,* in an effort to increase the rate at which L-malate was degraded during fermentation, was unsuccessful (Gao and Fleet 1995).

Saccharomyces cerevisiae strains vary widely in their ability to assimilate L-malate. Unlike *S. pombe*, *S. cerevisiae* lacks an active malate transport system and L-malate enters it by simple diffusion. Inside the cell, *S. cerevisiae*'s own constitutive NAD-dependent malic enzyme converts L-malate to pyruvate, which will be converted to ethanol and carbon dioxide under anaerobic conditions. Aerobically, malic acid is decarboxylated into water and carbon dioxide. Although the biochemical mechanism for malate degradation in

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S. cerevisiae is the same as in *S. pombe*, the substrate specificity of the *S. cerevisiae* malic enzyme is about 15-fold lower than that of the *S. pombe* malic enzyme (Volschenk et al. 1997). This low substrate specificity, combined with the absence of an active malate transport system, is responsible for the inefficient metabolism of malate by *S. cerevisiae*.

Several groups have explored the genetic engineering of wine yeast to conduct alcoholic fermentation and malate degradation simultaneously. In order to engineer a malolactic pathway in *S. cerevisiae*, the malolactic genes (*mle*S) from *Lactococcus lactis* (Ansanay et al. 1996; Denayrolles et al. 1995) and *Lactobacillus delbrueckii* (Williams et al. 1984) and the *mle*A gene from *O. oeni* (Labarre et al. 1996) were cloned and expressed in *S. cerevisiae*. The *mle*S gene encodes a NAD-dependent malolactic enzyme that converts L-malate to L-lactate and carbon dioxide (Denayrolles et al. 1995). However, these engineered strains could still not metabolize malate efficiently because there is no active malate transport system in *S. cerevisiae* (Volschenk et al. 1997). Malolactic fermentation was efficient only when the *L. lactis mle*S gene was coexpressed with the *S. pombe mae*1 gene encoding malate permease (Volschenk et al. 1997).

In the same way, an efficient malo-ethanolic *S. cerevisiae* was constructed by coexpressing the *mae*1 permease gene and the *mae*2 malic enzyme gene from *S. pombe* in *S. cerevisiae* (Volschenk et al. 2001). The unreliable bacterial malolactic fermentation could be replaced by a functional malolactic wine yeast, while a malo-ethanolic strain of *S. cerevisiae* would be more useful for the production of fruity floral wines in the cooler wine-producing regions of the world.

In contrast, the acidification of high pH wines produced in the warmer regions with wine yeast would be an inexpensive and convenient biological alternative. The formation of high levels of L-lactic acid by *S. cerevisiae* during alcoholic fermentation would help to reduce the pH. Lactic acid is widely used as a food acidulant because of its pleasant acidic flavor and its properties as a preservative. It also is present naturally in most fermented products, including wine, where it may be present in amounts of up to 6 g/l after malolactic fermentation (Dequin et al. 1999).

Natural *S. cerevisiae* strains produce only traces of lactic acid during alcoholic fermentation because of the inefficiency of the mitochondrial lacticodehydrogenases under fermentation conditions (Genga et al. 1983). In an attempt to redirect glucose carbon to lactic acid in *S. cerevisiae,* the lacticodehydrogenase-encoding gene from *Lactobacillus casei* and the bovine lacticodehydrogenase-encoding gene (Porro et al. 1995) were expressed in laboratory yeast strains. This construct was also introduced into eight wine yeast strains because the *L. casei* lacticodehydrogenase gene, expressed under control of the yeast alcohol dehydrogenase gene, converted 20% of the glucose into L-lactic acid (Dequin et al. 1999). Wines made with these engineered lactic acid-alcoholic fermentation yeasts were shown to be effectively acidified, but the fermentation rate was slower (Dequin et al. 1999).

6.6 Elimination of Phenolic Off-flavor

Phenolic acids (principally ρ-coumaric and ferulic acids), which are generally esterified with tartaric acid, are natural constituents of grape must and wine and can be released as free acids by certain cinnamoyl esterase activities during the winemaking process. Free phenolic acids can be metabolized into 4-vinyl and 4-ethyl derivates by different microorganisms in wine. These volatile phenols contribute to the aroma of the wine. *S. cerevisiae* contains the *PAD*1 (phenyl acrylic acid decarboxylase) gene, which is steadily transcribed in yeast. However, the activity of this enzyme is low. Phenolic acid decarboxylase from *B. subtilis* and ρ-coumaric acid decarboxylase from *Lactobacillus plantarum* display substrate inducible decarboxylating activity with phenolic acids. The *S. cerevisiae PAD*1 gene and both the *B. subtilis* ρ-coumaric acid decarboxylase (*pdc*) and phenolic acid decarboxylase (*padc*) genes were expressed in *S. cerevisiae* under the control of the *PGK*1 promoter (Smit et al. 2003). Industrial wine strains of *S. cerevisiae* with an interrupted *PAD*1 gene were also made. The overexpression of two bacterial genes, *pdc* and *padc*, in *S. cerevisiae* showed high enzyme activity in laboratory strains, whereas the overexpressed *PAD*1 did not result in any higher Pad1 enzyme activity than the control strain. Wine made with yeasts in which the *PAD*1 gene was disrupted and in which the *B. subtilis pdc* and *padc* genes were overexpressed contained higher levels of volatile phenols. It was also found that the overexpression of the *padc* gene in wine yeasts resulted in wine with elevated levels of favorable monoterpenes. This study paves the way for the development of wine yeast starter cultures for the production of optimal levels of volatile phenols, thereby improving the sensorial quality of wine.

6.7 Reduced Sulfite and Sulfide Production

Sulfur-containing compounds have a profound effect on the flavor of wine because of their high volatility, reactivity, and potency at very low threshold levels (Rauhut 1993). These substances are formed in grapes during ripening, while the dusting of vines with fungicides containing elemental sulfur provides another source. Sulfite is deliberately added to most wines as an antioxidant and antimicrobial agent during the winemaking process. However, health concerns and an unfavorable public perception of sulfite have led to demands for the restriction of its use and a reassessment of all aspects of sulfite accumulation in wine. The production of sulfur-containing compounds by wine yeast itself therefore has become a focal point of research.

The formation of sulfite and sulfide by wine yeasts has a considerable effect on the quality of wine. Unlike sulfur dioxide (SO_2), which has some beneficial effects when used properly, hydrogen sulfide (H_2S) is one of the most undesirable of yeast metabolites, since it causes an off-flavor reminiscent of rotten eggs above threshold levels of 50–80 g/l

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(Snow 1983). Sulfite is only formed from sulfate, while sulfide is formed from sulfate, sulfite, from elemental sulfur applied as a fungicide, and from cysteine (Rauhut 1993; Snow 1983). In addition to the yeast-strain effect, many other factors also affect sulfite and sulfide formation, including the nutrient composition of grape juice, the concentration of sulfate, must clarification, and the initial pH and temperature (Rauhut 1993).

Sulfur is essential for yeast growth and *S. cerevisiae* can use sulfate, sulfite and elemental sulfur as sole sources. Defects in sulfate uptake and reduction, which are normally regulated by methionine via its metabolites methionyl-tRNA and *S*-adenosylmethionine, can result in excessive sulfite and sulfide production (Henschke 1997). Sulfate is transported into yeast cells by the *SUL*1 and *SUL*2-encoded high-affinity sulfate transporter proteins, whose expression is regulated by the *SUL*3-encoded transcriptional factor (Cherest et al. 1997). Sulfite enters the cells either as sulfur dioxide via an active carrier-mediated process, by simple diffusion, or by active transport, whereas the efflux of sulfite is mediated by the *SSU*1-encoded sulfite pump when it is activated by the *FZF*1 gene product (Park and Bakalinsky 2000). The formation of sulfite and sulfide depends on four key enzymes (Donalies and Stahl 2002): Once inorganic sulfate is taken up by the yeast cells, it is activated into adenosylphosphosulfate (APS) by the *MET*3-encoded ATP sulfurylase. In the following step, sulfate is again phosphorylated by the *MET*14-encoded APS kinase, yielding phosphoadenosylphosphosulfate (PAPS). The activated sulfate is then reduced by the *MET*16-encoded PAPS reductase, generating sulfite and phosphoadenosylphosphate (PAP). Finally, sulfite is reduced to sulfide by sulfite reductase, which is encoded by *MET*10 and *MET*5.

The transcription of the *MET* genes that are involved in the sulfur amino acid pathway is repressed when methionine, *S*-adenosylmethionine (SAM), homocysteine or higher concentrations of cysteine are added to the culture medium. Six different regulatory factors regulate the expression levels of the *MET* genes, namely the inhibitor Met30p and the activators Cbf1p, Met28p, Met31p, Met32p, and Met4p (the main transcriptional activator) (Donalies and Stahl 2002). With the exception of *MET*16, the promoters of the *MET* genes do not contain any binding sites for the Gcn4p activator and are therefore not subject to the general control of amino acid biosynthesis.

Sulfite formation as part of amino acid biosynthesis in *S. cerevisiae* is very complex and highly regulated. This makes it difficult to manipulate sulfite formation in yeast when the possible differences between naturally produced and artificially added sulfite are investigated. An industrial strain of *S. cerevisiae* in which both alleles of *MET*14 were disrupted produced significantly lower levels of sulfite, but there was no qualitative difference between yeast-derived and artificially added sulfite with respect to flavor stability (Donalies and Stahl 2002). Conversely, the overexpression of cloned copies of the *MET*14 and *MET*16 genes resulted in a two- to three-fold increase in sulfite formation (Donalies and Stahl 2002). It was also found that the inactivation of *MET*10 resulted in a distinct increase in sulfite production, but the cells became methionine auxotroph (Hansen and Kielland-Brandt 1996). The overexpression of *SSU*1 yielded a slight increase in sulfite accumulation, whereas the overexpression of *SSU*1, together with *MET*14, increased sulfite formation as much as ten-fold (Donalies and Stahl 2002).

H_2S is formed by yeast during fermentation, largely in response to nutrient depletion, especially assimilable nitrogen and possibly vitamins such as pantothenate or pyridoxine (Henschke 1997). If the H_2S sequestering molecules *O*-acetylserine and *O*-acetylhomoserine are absent, as caused by nitrogen starvation, free H_2S accumulates and diffuses from the cell (Jiranek et al. 1995a). Some grape varieties (e.g., Riesling, Chardonnay and Syrah) tend to have a low nitrogen content caused by soil type and vintage conditions. This problem often can be suppressed by adding nitrogen (typically in the form of DAP) during fermentation. However, there have been reports that impaired membrane transport function and intracellular deficiency of certain vitamins can also cause H_2S accumulation (Henschke 1997).

The addition of a high level of SO_2 to the must shortly before inoculating with yeast, and the yeast strain involved, can also affect the amount of H_2S produced. Certain yeasts reduce sulfate and SO_2 to H_2S more readily when deprived of nitrogen in a futile effort to synthesize and supply sulfur-containing amino acids to the growing yeast cell (Henschke and Jiranek 1993; Jiranek et al. 1995a–c). Adding ammonium salts prevents H_2S accumulation in wine, not by stopping its formation, but by enabling the yeast to synthesize amino acid precursor compounds that react with H_2S to form sulfur-containing acids (Henschke and Jiranek 1993). As a result of higher fermentation temperatures in hot climate red wine production, yeast cells use more nitrogen during rapid fermentations and tend to develop sulfidic smells. Fortunately, H_2S is highly volatile and can usually be removed by the stripping action of CO_2 produced during these rapid high-temperature fermentations (Henschke 1997). However, H_2S formed towards the end of, or after, fermentation can react with other wine components to form mercaptans, thiols, and disulfides. These have pungent garlic, onion, and rubber aromas (Henschke and Jiranek 1993).

There is a great difference among yeast strains regarding their ability to produce sulfite and sulfide (Henschke and Jiranek 1993). This fact can be exploited by selecting or developing a wine yeast strain that either will produce less H_2S or will retain most of the H_2S produced intracellularly. Several laboratories have also demonstrated that yeast strains with low H_2S production and improved winemaking properties can be bred by hybridization. However, it appears that the most effective way in which wine yeast strains with a decreased ability to produce sulfite and sulfide can be developed is by disrupting the *MET*14 gene (Donalies and Stahl 2002).

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7 CONCLUSIONS

In the past century, the availability of pure culture yeast has improved product quality and reproducibility in wine fermentations. However, there is not a single wine yeast strain that possesses an ideal combination of enological characteristics that are optimized for the task set by today's leading winemakers. With new developments in modern winemaking, an urgent need has arisen to modify wine yeast strains in order to take full advantage of technology and to meet the demands of both the producer and the consumer. Therefore, the overall aim of the genetic improvement of wine yeast strains extends far beyond the primary role of wine yeast to catalyze the rapid and complete conversion of grape sugars to alcohol and carbon dioxide without distorting the flavor of the final product. The new generation of "designer" starter cultures of *S. cerevisiae* must possess a wide range of other properties that differ with the type and style of wine to be made and the technical requirements of the winery (Grossmann and Pretorius 1999; Pretorius 1999; 2000; 2001; Pretorius and Bauer 2002).

It is clear that, in the past few years, considerable progress has been made in developing new wine yeast strains. However, while the scientific case for the use of genetically modified and improved wine yeast starter culture strains is strong and persuasive, recombinant wine yeasts have not, as yet, been used commercially. Several factors relating to scientific, technical, economic, marketing, safety, regulatory, legal, and ethical issues have hindered, and may in the near future continue to hinder, the successful commercialization of genetically modified wine yeasts (Walker 1998). These factors include the uncertain, lengthy, and costly process of acquiring patent rights, obtaining government approval and gaining consumer acceptance of GMOs. Each of these challenges is daunting in its own right; however, the magnitude of their complexity is often amplified by the groundless, scaremongering exaggerations of nongovernmental "watchdog" organizations. They talk about the unsafeness of so-called "Frankenfood" and the environmental havoc that could potentially result from the inadvertent production of new pathogenic organisms, uncontrolled transfer of modified genes to other organisms outside the laboratory and the colonization of the environment by harmful GMOs that put the planet's biodiversity at risk. This irrational and confusing situation is further clouded by political meddling from the vested interests of economic and agricultural protectionism.

It therefore is essential to provide the confused consumer with scientifically sound information and to convince the public that the staggering potential benefits far outweigh the small risks related to the use of GMOs for the production of a wider choice of safe, high-quality, affordable foods, and beverages. The growing acceptance of the concept of "substantial equivalence" is a faint sign on the horizon that some progress is being made in this regard. The definition of substantial equivalence is widely used in the determination of safety by comparison with analogous conventional food and beverage products (Smith 1998). When substantial equivalence can be demonstrated, no further safety considerations usually are necessary. When substantial equivalence is not convincingly shown, the points of difference must be subjected to further scrutiny regarding safety.

When one steps back from the economic phenomena of the past few hundred years, one can see that economies go through lifecycles. The *Industrial Economy* has been with us for several centuries and, in terms of resources, it was driven by "the economics of scarcity." Everything that fueled the economy was in short supply and available to only a few nations. The new *Information Economy*, driven by communications and computer technologies (information, knowledge, skills and ideas), has brought more economic transformation in the past few decades than was brought by the Industrial Economy in the previous centuries. Clearly, the Information Economy was built on the successes of the Industrial Economy and then leapfrogged the economic impact. Thanks to the Internet, information is no longer a scarce resource. Thus, the exploding Information Economy has really created "the economics of plenty." However, we are already sensing the rumbling beyond the horizon of the Information Economy; genetic engineering is the science of the 21st century and could turn out to be one of the most spectacular bull-runs in history (Pretorius 2000; Vivier and Pretorius 2000). This so-called *BioTech Revolution* or *Bioeconomy* may well turn out to be more powerful than the Information Economy ever was. In fact, the Information Economy may turn out to be the first phase of the *Bioeconomy*.

With their broad experience in yeast-based fermentations, winemakers are well placed to explore the new opportunities offered by the exciting age of gene technology and bioinformatics. In particular, well-considered, market-driven research and business strategies focused on information obtained from the analysis of the entire genomes, transcriptomes, proteomes and metabolomes of wine yeast strains promise to unleash the full potential of genetic engineering. This could provide the wine industry with the quantum leap that is desperately needed to close the current, widening gap between wine production and wine consumption, and to propel the industry into the new era of the so-called "Bioeconomy." It is an opportunity that the wine industry cannot afford to miss.

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18

Fungi in Brewing: Biodiversity and Biotechnology Perspectives

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1 INTRODUCTION

One of the main milestones of Old World civilization was reached when cereals became the central nutritional source of humans. This change in diet provided our ancestors, nomadic hunters and collectors of fruits, with the option to settle down and build a more permanent home. This farming society in turn formed the basis for the invention of two of the oldest food technologies, bread baking and brewing, the latter providing early societies with a nutritious and relatively safe drink that would also work as an intoxicant. Since then these two technologies have become an integral part of humankind's daily life and activities. The central biological agent of both baking and brewing are unicellular fungi, so-called yeast. In the following, we will focus on the role of yeast in beer brewing, ancient as well as contemporary, and describe the biodiversity of the organisms used. The biological processes underlying the fermentation process will be discussed in the context of the targeted breeding of these organisms. Finally, the perspectives for the usage of yeast in brewing in the future will be briefly discussed.

2 GRAIN INTO GOLD: THE INVENTION OF BEER

How was the idea of making a sparkling alcoholic beverage out of germinated barley grains conceived? The most likely answer is: through a series of fairly random events. Malting probably first arose as a way of improving grain palatability and nutritional value, as a germinated grain contains highly assimilable sugars that doubtlessly were more agreeable to the human palate than raw grains. Malt mixed with water gave a liquid that could easily undergo spontaneous fermentation, and thus, in principle, the beer was invented. It is well established that beer brewing was known and widely used in ancient times, as early as 3000 BC in Mesopotamia (see Corran 1975 for a review of beer brewing history). Quite recent studies (Samuel 1996) indicate that in Egypt during "The New Kingdom," more than 3000 years ago, sophisticated regimens for the malting of barley and the primitive wheat *emmer* existed, much like those used today, and that yeast was involved in the fermentation process. In comparison, beer brewing in Europe is a rather new technological landmark that appears to be acquired from the Middle East independently by Germanic and Celtic tribes around the 1st century AD (Corran 1975). From then on, development of the brewing processes took place in England/Britain and in the European heartland, in particular Bohemia and Germany. While on the British Isles the tradition of ale brewing was taken to perfection, in mainland Europe ale brewing was developed into lager brewing (see later). An important technological milestone in beer brewing was the first pure culturing of brewing yeast by Emil Christian Hansen at the Carlsberg Laboratory, and use of pure cultures for production scale brewing (Hansen 1883; Hansen 1908). Shortly after that, the use of pure yeast cultures was adopted worldwide, first in lager brewing and subsequently in ale brewing. While mixed cultures of several yeast strains are being used quite widely in ale brewing, spontaneous fermentation is used at an industrial scale only in Belgian *lambic* brewing.

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3 THE PHYSIOLOGY OF BEER PRODUCTION

The processes that led to the fabrication of one of today's most important consumables, may be divided into five main stages (see Hough et al. 1982 for a thorough description of technical and scientific aspects of beer brewing). (a) Firstly, barley grains of a special malting variety are germinated (the "malting" process). After steeping the grains with water, sprouting is accompanied by the biosynthesis and activation of endogenous enzymes, which are transported to the grain endosperm. Cell wall material is broken down at this stage. (b) Next, the malt is dried and milled to powder, which is then immersed in water. (c) Now the suspension is heated in stepwise increments in temperature to activate carbohydrate- and protein-degrading barley enzymes, and the "sweet wort," or filtered malt extract, is boiled with hops or hop extract to produce the final brewer's wort. The wort contains low molecular weight sugars, most notably maltose, and forms an ideal growth medium for propagated brewing yeast, which is now added. (d) The primary fermentation proceeds for a number of days, depending on the type of beer produced. (e) Maturation, or secondary fermentation, follows, usually to allow the remaining yeast cells to obtain desired flavors and/or to get rid of off-flavors. This scheme is generalized, and several variations are seen: addition of roasted malt to produce dark beers, use of wheat for German *weizenbier* and Belgian *Witbier*, addition of other sources of starch, such as maize or rice (common in lager production), or use of bacterial fermentation as part of the process (Belgian *lambic* beer). The species and varieties of yeast employed for the primary fermentation is of huge significance for the outcome of the process, as many important beer flavor components are yeast metabolites: alcohols, aldehydes, esters, thiols etc. Traditionally, the fermentation process is carried out by the so-called "top-" or "bottom-fermenting" brewing strains. Ales are fermented in flat, open vessels with top-fermenting yeasts, characterized by their adherence to carbon dioxide bubbles, which will rise to the surface at the end of fermentation. The yeast is harvested from here for use in consecutive fermentations. On the contrary, lager beer (and other "bottom") fermentations are usually carried out in tall cylindroconical vessels, often containing about half a million liters, using "bottom-fermenting" yeasts; in these the yeast will sediment to the bottom after fermentation, from where it is harvested. In lager brewing, the yeast influences the quality of the final product at two stages: the primary and secondary fermentations. After addition of the yeast to the cooled wort, the primary fermentation proceeds at 8–15°C for up to 10 days. Here alcohol, carbon dioxide, and much of the aroma are formed. Secondary fermentation or "lagering," a maturation period of 1–3 weeks at a slightly lower temperature, follows to obtain the final taste. During these processes many factors influence the characteristics of the final beer, including the genotype of the lager yeast variety used. This forms the basis for research into the possible alterations and performance of the yeast. Briefly, the following yeast capabilities are of particular interest for the brewer and breeder: uptake of carbohydrates, amino acids, and ions; primary metabolism, i.e., ability to turn carbohydrate into energy and alcohol; secondary metabolism, leading to the formation of aroma compounds; sedimentation characteristics, and stress response, vitality, and viability.

4 YEAST BIODIVERSITY

Yeasts constitute a quite divergent group of fungi, comprising representatives of *Ascomycetes* and *Basidiomycetes* (Kurtzman and Fell 1998). However, a majority of the characterized brewing yeast isolates belong to the ascomycetous genus *Saccharomyces*. In addition, several "traditional" beers involve contributions from a range of *Brettanomyces/Dekkera*, *Candida*, *Saccharomycopsis*, and *Schizosaccharomyces* yeasts (Van Oevelen et al. 1977; Verachtert and Dawoud 1990).

The genus *Saccharomyces* has a taxonomic history of over 150 years. During this time period, the isolates and species belonging to this genus have been frequently changed and/or renamed, mainly depending on the availability and development of methods for yeast characterization and the mood of the involved taxonomists (Barnett 1992). At present, over twenty species belong to the *Saccharomyces* genus but it is expected that in the coming years, a number of new species will be isolated and characterized (Kurtzman and Robnett 1998). The genus *Saccharomyces* includes *petite*-positive and *petite*-negative yeasts. The latter group has only one member, namely *S. kluyveri*, which cannot yield respiratory deficient mutants, characterized by large mitochondrial deletions and rearrangements. It should be stressed that a majority of other yeasts, including closely related genera such as *Kluyveromyces*, are also *petite*-negative. Therefore *S. kluyveri* should perhaps rather be considered as a member of a different genus (Møller et al. 2001; Piskur et al. 1998). The *petite*-positive *Saccharomyces* yeasts are divided into two sub-groups: *sensu stricto* (including *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* syn. *carlsbergensis*) and *sensu lato*. So-called "top fermentations" are usually carried out by *S. cerevisiae* strains, and "bottom fermentations" by *S. pastorianus* syn. *carlsbergensis* strains (Pedersen 1986a).

A number of studies have contributed to elucidating the organization of the *Saccharomyces* genomes, culminating with the determination of the complete genome sequence of *S. cerevisiae* (Goffeau 1996). The closely related *Saccharomyces sensu stricto* species have relatively uniform karyotypes consisting of sixteen chromosomes. On the other hand, the karyotypes of the *Saccharomyces sensu lato* species exhibit much heterogeneity and the number of chromosomes is usually below 16, except for *S. exiguus*, which also has 16 chromosomes (Petersen et al. 1999). However, even among isolates of the same species natural polymorphism can often be observed (Piskur et al, manuscript in preparation).

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Mitochondrial DNA molecules (mtDNA) of *Saccharomyces* yeasts fall into two groups: the *sensu stricto* mtDNAs with a size above 60 kb and similar gene order configurations, and the *sensu lato* mtDNAs, which are smaller than 60 kb and show a higher degree of phylogenetic and gene order diversity (Groth et al. 2000).

A special class among *Saccharomyces* yeasts comprises hybrids. Occasionally hybridization between two yeast species occurs in nature, and sometimes results in viable hybrids. The most well-described example is actually the lager brewing yeast, *S. pastorianus* syn. *carlsbergensis*. This yeast is an allotetraploid hybrid between two *Saccharomyces sensu stricto* species, *S. cerevisiae* and an unknown *S. bayanus*-related yeast (Hansen and Kielland-Brandt 1994; Pedersen 1986b). While nearly complete sets of both the parental chromosomes are preserved in *S. pastorianus*, the mtDNA molecule originates from the non-*S. cerevisiae* parent (Piskur et al. 1998). Several other yeast isolates have been recently shown to be hybrids (de Barros Lopes et al. 2002; Masneuf et al. 1998), and they often combine the fermentation characteristics of both parents (Masneuf et al. 1998). From the genetic point of view perhaps the most interesting hybrid is the cider brewing isolate CID1, a natural hybrid of three species, originating from a home brewery in Brittany. Two of the parents are ubiquitous and contributed parts of the nuclear genome, while the third, which has so far been found only in Japan, contributed the mtDNA molecule (Groth et al. 1999). It seems that horizontal transfer of genetic material can contribute a significant additional source of genetic variation within *Saccharomyces* yeasts. Because hybridization between two different species is relatively easy to perform under laboratory conditions (Marinoni et al. 1999), it represents a useful tool to generate novel brewing strains with improved characteristics.

Another yeast genus, *Brettanomyces/Dekkera*, which also possesses good brewing characteristics (van Oevelen et al. 1977), is from the phylogenetic point of view not closely related to *Saccharomyces* yeasts. *Brettanomyces/Dekkera* yeast species are involved in brewing of certain Belgian beers, but so far they have only been poorly characterized. These yeasts are *petite*-positive, at least some of them can also grow anaerobically, and they are good ethanol producers (Verachtert and Dawoud 1990). While their mtDNAs have been characterized and shown to vary in size from 28 to 101 kb (Hoeben et al. 1993), the nuclear genes and chromosomes are not well characterized. These yeasts undoubtedly represent a potential genetic source for development of novel brewing strains.

5 HOW DID *SACCHAROMYCES* YEASTS BECOME GOOD BREWERS?

The basic biochemical and physiological requirement of a brewing yeast is to degrade the carbon source only to the C_3 and C_2 components, including pyruvate and ethanol, respectively, and not completely to CO_2, even in the presence of oxygen. In other words, the energy for growth should be provided by the glycolysis and fermentation pathways, and not by the oxidative respiration pathway. This requirement relies on development of a "glucose-repression" circuit, in which the presence of C6 carbohydrates, such as glucose, represses the respiration (Polakis et al. 1965). Therefore, in the presence of glucose, pyruvate originating from glycolysis is directed only, or mainly to the fermentation pathway. The most prominent feature of *Saccharomyces* yeasts is the ability to rapidly convert sugars to ethanol and carbon dioxide under both aerobic and anaerobic conditions. Under the aerobic conditions respiration is possible, but the *Saccharomyces* yeasts still exhibit predominantly alcoholic fermentation (Møller et al. 2001; Pronk et al. 1996). This occurrence of fermentation under aerobic conditions is referred to as the Crabtree-effect (Swanson and Clifton 1948).

Another important step during the evolution of "the basic fermentation properties," is the survival of yeast without oxygen. This is, in a sense, an additional security checkpoint to ensure that the C_3 and C_2 compounds are not converted further to CO_2, even when the sugar pool is already depleted. While a majority of yeasts cannot grow in the absence of oxygen, *Saccharomyces* and *Brettanomyces/Dekkera* can (Andreasen and Stier 1953; Møller et al. 2001; Subik et al. 1974). In short, the *Saccharomyces* yeasts fulfill both basic requirements to be good brewers. However, it is not clear when and how the progenitor of *Saccharomyces* yeasts developed basic brewing properties.

The complete sequencing of the yeast *S. cerevisiae* supplied the first eukaryotic genome sequence, and also provided a tool to study the yeast evolutionary history. Many of the eukaryote genomes that have been sequenced so far, show a high degree of gene redundancy. This redundancy is a consequence of gene duplication, a molecular mechanism that is believed to play a major role in biological evolution (Ohno 1970). Upon duplication and sequence divergence each of the two copies can become functionally specialized. In the genomes of the fruit fly and the worm *Caenorhabditis elegans*, as well as two ascomycetous yeasts, *Candida albicans* and *Schizosaccharomyces pombe*, most of the duplicated genes are dispersed. In contrast, the arrangement of duplicated genes in *S. cerevisiae* and the thale cress *Arabidopsis thaliana* coincides with large segmental duplications (Arabidopsis Genome Initiative 2000; Wolfe and Shields 1997). A whole genome duplication or a series of smaller duplications or a combination of both mechanisms have been proposed as the origin for the *S. cerevisiae* large duplicated regions (Langkjær et al. 2000; Llorente et al. 2000; Wolfe and Shields 1997). Whatever the mechanism, the rationale for the *Saccharomyces* genome large segmental duplications remains unresolved.

The majority of ascomycetous yeasts are strictly aerobic and cannot be propagated at low oxygen levels. However, several of them, such as *Kluyveromyces lactis* (Wesolovski-Louvel et al. 1996), which is a relatively close relative of *S. cerevisiae*, do not require oxygen for energy metabolism.

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In this case, the oxygen dependence is at least partially due to the dependence of several biochemical pathways, such as biosynthesis of sterols, pyrimidines, and deoxyribonucleotides, on the presence of molecular oxygen (Andreasen and Stier 1953; Chabes et al. 2000; Nagy et al. 1992). Based on the comparison of the modern yeast genera, it is likely that the common progenitor was fully dependent on the presence of oxygen. However, upon diversification, some of the yeast lineages progressively decreased their dependence on oxygen. While some of the genera closely related to *Saccharomyces* have only moderately decreased the dependence on oxygen, the *S. cerevisiae* lineage has become almost oxygen independent. This evolutionary step, which possibly took place 100–200 million years ago, could require a major remodeling of the yeast metabolism and regulatory mechanisms (Piskur 2001). This may have coincided with the radiation of the modern plants, app. 200 million years ago, and the appearance of larger quantities of "free" monosaccharides and "anaerobic conditions" in some of the organs of higher plants. Segmental duplication(s) in the *S. cerevisiae* lineage may have provided the basis for specialization of a number of duplicated genes and their products, enabling their optimal function even in the absence of oxygen. The resulting lineages apparently had a competitive advantage: they could grow fast in the absence of oxygen, and they produced ethanol, which may have been toxic for competitors. The newly acquired phenotype was also one of the reasons that the *Saccharomyces* yeasts later became one of humankind's favorite pets, responsible for the production of a robust alcoholic product. To turn this product into a tasty drink, however, yeast selection has been necessary, and later on, breeding of yeast strains.

6 METHODOLOGY: HOW TO BREED

While selection for genetic adaptation to man-made beer-fermentation conditions undoubtedly provided the brewing yeast with the basic abilities to produce beer, further breeding may certainly be beneficial or even necessary. Despite the genetically complicated nature of *S. carlsbergensis* lager yeast, classical breeding of *S. carlsbergensis* is possible. Gjermansen and Sigsgaard (1981) described the isolation of lager yeast spore segregants with the ability to mate. A few of these could, upon crossing, form a hybrid yeast that performed almost like the parental lager yeast in pilot fermentation experiments. Maters derived from an irregular allotetraploid are expected to be alloploid, which has indeed been observed for several chromosomes (Johannesen and Hansen 2002; Hansen et al. 2002; Hansen and Kielland-Brandt 1996a; Hansen and Kielland-Brandt 1996b; Tillgren et al. 1986; Nilsson-Petersen et al. 1987), and possibly aneuploid (Hoffman 2000). Hybrids formed from an array of these maters form quite a heterogeneous population of potential brewing strains (Gjermansen and Sigsgaard 1981), some of which could be better suited to new brewing conditions. The use of maters from other yeasts (Bilinski et al. 1987; Bilinski and Casey 1989), would allow the introduction of desired characters from a nonbrewing yeast into well-performing brewing yeasts. Due to the tetraploid genetic background of lager yeasts, it is generally impossible to obtain recessive mutations in this group, but recessive mutants of supposed allodiploid lager yeast spore clones (Gjermansen and Sigsgaard 1981) have been described (Gjermansen 1983). Direct selection of dominant mutants in brewing yeasts can obviously be performed, provided an adequate selection system has been developed.

Other methods for breeding of lager yeast without the use of recombinant gene technologies have also been described. One is "rare mating," in which characterized haploid maters are mixed with a population of brewing yeasts, and rare hybrid products are selected (Tubb et al. 1981). By another technique, protoplasts of cell wall-stripped yeasts are fused, using either polyethylene glycol (PEG) and calcium ions, or electrofusion, thus avoiding the question of the mating type altogether (Russell and Stewart 1979; Urano et al. 1993a,b).

Most of the techniques described so far implicate the transfer of several or many genes, and in rather unpredictable ways. This may be an advantage in some cases, but in directed breeding projects, a greater degree of precision is needed. Modern recombinant gene technology provides the yeast breeder with the desired means.

Historically, transformation of brewing yeast with recombinant DNA relied on the use of 2 μm-based plasmids (Gjermansen 1983; Henderson et al. 1985). While such plasmids have a high copy number and thus give a high expression, plasmid systems are usually hampered by mitotic loss (Romanos et al. 1992). On the other hand, integration into the brewing yeast genome ensures a quite high degree of mitotic stability, depending on the exact method employed. While all integration methods rely on recombination between homologous sequences on the integration plasmid and the yeast chromosome, the choice of technique depends on many factors: transformability of the brewing yeast strain in question, whether one wants to introduce, alter or inactivate a gene, and the availability of selection markers. Three methods are in common use: (a) Simple integration of a complete plasmid containing yeast sequences, (b) single-step integration of a DNA fragment, containing yeast sequences at both ends (Rothstein 1991), and, (c) the two-step substitution procedure (Scherer and Davis 1979). The advantage of the first procedure is its simplicity, but since adjoining homologous sequences are present after integration, mitotic stability is not complete (Romanos et al. 1992). Single-step integration of a DNA fragment is very simple, but will usually result in the permanent insertion and concomitant expenditure of a genetic marker. The two-step procedure, on the other hand, relies on a single initial recombination event and ends with the removal of homologous yeast sequences and the selection marker, and it may be used for integration of heterologous genes or homologous genes with altered structure, or for removing genes or parts of genes. The principle relies on homologous recombination at one site, thus integrating the whole nonreplicating plasmid. A dominant

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selection marker identifies the integrant. Subsequently, a secondary recombination event at a site distal to the heterologous or altered homologous gene sequences results in the excision of plasmid sequences, including the selection marker. If inactivation is desired, a deletion of the gene in question remains between the two sites of recombination. A virtue of this procedure is that vector sequences are completely removed, leaving just the desired genetic change. Due to the polyploid nature of lager yeast, it may be necessary to carry out the procedure on two or more homologous chromosomes, in order to avoid "repair" of the alteration by mitotic recombination. In the case of removal of all activity of a wild type yeast gene, copies on the divergent chromosomes must also be inactivated. The historic disadvantage with this procedure was that the search for "loop-outs," the results of the secondary, rare recombination event, was very laborious. Recently, however, the inclusion of counter-selection markers on integration plasmids has been described (Olesen et al. 2000), enabling easy selection for "loop-outs."

Regardless of the method, dominant gene markers are needed for the transformation of polyploid yeasts. In early experiments, the *CUP1* marker, giving copper resistance, was used with 2 μm-based plasmids (Henderson et al. 1985). As the acquired resistance depends on high-copy number expression, this marker is not used for integration experiments. The *SMR1* sulfometuron methyl (SM)-resistant *ILV2* mutant allele causes dominant SM resistance by encoding a form of acetohydroxy acid synthase resistant to the herbicide (Casey et al. 1988; Falco and Dumas 1985). Bacterial resistance genes expressed by yeast promoters may be the most promising selection tool for industrial use, and combined with the two-step integration procedure, the use of DNA foreign to the brewing yeast should in principle not pose problems. A widely used marker is the geneticin (G418) resistance marker (Yocum 1986), and other possibilities include the glyphosate marker (Kunze et al. 1989), and the nourseothricin and hygromycin markers (Goldstein and McCusker 1999).

Thus, a diverse array of methods exists for directed genetic modification of lager yeast and other brewing yeasts, and in the following we will describe a range of current brewing research interest areas, as well as the implementation of the described breeding methods in attempts to improve the yeast genotype in relation to these areas. Recent reviews in the field include Abbott et al. (1993), Brandt et al. (1989, 1995), Dequin (2001), Hammond (1995), Hansen and Kielland-Brandt (1996c), Kielland-Hinchliffe (1991), Kielland-Brandt (1994a,b), Penttilä and Enari (1991), and Polaina (2002).

7 ROOM FOR IMPROVEMENT

7.1 Feeding the Beast: Carbohydrate Degradation

When producing the average beer, the brewer wants the yeast to use up as much substrate, i.e., carbohydrate, as possible, as quickly as possible. Wort carbohydrates fall in two main groups: nonfermentable and fermentable. The former group is mainly composed of dextrins of varying length, and constitutes ca. 25% of total all-malt wort carbohydrate content. The fermentable group consists of the poly- and oligosaccharides maltose, glucose, sucrose, fructose, and maltotriose. Due to catabolite repression of the genes for maltose uptake and utilization and to glucose-mediated inactivation of the maltose and maltotriose transporter proteins, utilization of the maltose and maltotriose does not start until app. 50% of the wort glucose has been used (Stewart et al. 1983). While this phenomenon may be of lesser importance in low-gravity brewing, it may pose problems in high-gravity or super-high gravity brewing, in which high amounts of glucose are present. A few experiments have been performed in order to abolish the glucose-dependent lag time of maltose and maltotriose utilization. Stewart et al. (1985) found that ale brewing yeast strains that were resistant to the nonmetabolizable glucose analogue 2-deoxyglucose were able to metabolize maltose and maltotriose, even in the presence of high concentrations of glucose. This would accelerate complete fermentation of worts enriched for glucose. A more targeted approach was presented by Kodama et al. (1995): the genes for the α-glucosidase (maltase), the maltose transporter, and the positive regulator for these two were all overexpressed in lager yeast during high gravity brewing, from yeast promoters not repressed by glucose. Somewhat surprisingly, only overexpression of the gene for the transporter, *MALT*, had an impact: the speed of maltose removal from the wort was significantly increased, accompanied by a corresponding decrease in the total fermentation time. It is currently not known if maltotriose uptake can be improved in a similar way.

Let us now turn our attention to 25% of the wort carbohydrates that are nonfermentable, including the dextrins. These pose a problem when the brewer's goal is a low calorie beer, a type of product that is very popular in some countries. They also represent an unused ethanol precursor. Dextrins are breakdown products of the malt starch, and are usually branched oligosaccharides, containing both α-1,4- and α-1,6-glycosidic linkages. Exo-amylolytic breakage of such bonds from the nonreducing ends of the polysaccharides can be catalyzed by glucoamylases with α-1,4 hydrolytic activity, with the release of glucose as a result. Some glucoamylases also possess some debranching α-1,6 hydrolytic activity. α-Amylases, on the other hand, catalyze endoamylolytic cleavage of α-1,4 linkages, at a rather random basis, with the consequent release of oligosaccharides. If they were present together, these two types of amylolytic enzymes would quickly reduce most of the remaining dextrins in beer to fermentable carbohydrates. Indeed much low calorie beer today is being produced by the addition of crude microbial preparations containing relevant enzymatic activities.

In attempts to obtain somewhat more elegant and targeted solutions to the dextrin problem, much research has focused on constructing brewing yeasts that can secrete active

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Table 1 Biotechnological approaches to accomplish dextrin degradation during beer fermentation

Enzyme	Gene	Methodology	Improvements/Comments	Reference
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus STA* (*DEX1*)	Rare mating and backcrossing of brewing yeast to *S. cerevisiae* var. *diastaticus*.	Amylolytic yeast with good fermentation abilities.	Emeis 1971
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus STA* (*DEX*)	Rare mating and backcrossing of brewing yeast to *S. cerevisiae* var. *diastaticus.*	No phenolic off-flavor but only 25% utilization of dextrins.	Tubb et al. 1981
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus STA* (*DEX*)	Protoplast fusion between donor yeast and brewing yeast.	Clearly amylolytic hybrid yeasts with good fermentation performance. Production of phenolic off-flavor.	Freeman 1981
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus STA2* (*DEX1*)	High copy (2 μm) expression. *CUP1* as selection marker.	26–30% utilization of dextrins. Impaired fermentation rate.	Meaden and Tubb 1985; Perry and Meaden 1988
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus STA2* (*DEX1*)	"All yeast" high copy (2 μm) expression. *PGK1* promoter and *CUP1* as selection marker.	Clearly amylolytic yeast and good fermentation performance. Relatively good plasmid instability.	Vakeria and Hinchliffe 1989
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus STA2* (*DEX1*)	"All yeast" high copy (2 μm) expression. Yeast *PGK1* or *GPD1* promoters and *CUP1* selection marker.	Same degree of attenuation as with addition of commercial glucoamylase during fermentation. Plasmid stable and good yeast growth rate with the *GPD1* promoter.	Vakeria et al. 1996
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus STA1* (*DEX2*)	Low copy plasmid expression, using the native *STA1* promoter for expression and the G418 selection marker	Some decrease in beer dextrin concentration. 3.6% increase in beer ethanol content.	Sakai et al. 1989
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus STA1* (*DEX2*)	High copy (2 μm) expression or single-step double recombinatorial integration into rDNA. *ADH1* promoter and *CUP1* selection marker.	Significant decrease in beer dextrin concentration with both strategies. App. 10% increase in beer ethanol content with integration strain.	Park et al. 1990
Glucoamylase (α-1,4)	*S. cerevisiae* var. *diastaticus* mutant *STA1* (*DEX2*)	High copy (2 μm) expression using native mutant gene promoter and *CUP1* as selection marker.	Some increase in attenuation. Glucoamylase gene not glucose repressed.	Kim et al. 1994
Glucoamylase (α-1,4; α-1,6)	*A. niger* GA	Two-step stable integration (multiple copies) into *HO*. Yeast glycolytic promoter and *SGL* secretion signal.	Some decrease in beer dextrin content.	Yocum 1986
Glucoamylase (α-1,4; α-1,6)	*A. niger* GA	Two-step stable integration (multiple copies) into *HO*. Yeast glycolytic promoter and *SGL* secretion signal.	Almost half of beer dextrins removed. 20% increase in beer ethanol content.	Gopal and Hammond 1992
Glucoamylase (α-1,4; α-1,6)	*S. occidentalis* GAM1	High copy (2 μm) expression or integration. Yeast *ADH1* promoter *SMR1* selection marker.	Some dextrin degradation. Glucoamylase is heat labile, thus destructed by pasteurization.	Lancashire et al. 1989

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glucoamylases, either from *Saccharomyces* varieties or from other fungi. The results of pertinent studies, which cover a period of 30 years, are summarized in Table 1. Together, the various experiments performed have solved most of the problems associated with the construction of an amylolytic brewing yeast. Initial problems caused by the linkage of dextrin-fermenting ability with the production of phenolic off-flavor by the yeast (due to the presence of the *PAD1*/*POF1* gene (Tubb et al. 1981) became obsolete with modern molecular breeding techniques becoming available. It should, however, be noted that the *PAD1* gene can be removed in the *S. cerevisiae* var. *diastaticus STA/DEX* donor yeast, by classical genetic techniques (Russel et al. 1983). Good results with dextrin fermentation and plasmid stability were gained using episomal (2 μm) plasmid expression of various glucoamylase genes, but the integrative approaches (Lancashire et al. 1989; Park et al. 1990; Yocum 1986) seem to be more promising. Thus, Park et al. (1990), obtained significant dextrin fermentation and a 10% increase in ethanol production by integration of *S. cerevisiae* var. *diastaticus STA1*(*DEX2*) in multiple copies into the genomic rDNA genes. The *Aspergillus niger* GA gene was inserted in all three genomic copies of the brewing yeast *HO* gene (Gopal and Hammond 1992; Yocum 1986), and this resulted in an impressive 50% decrease in dextrin content and 20% increase in ethanol production. This glucoamylase is thermostable, and hence not inactivated by pasteurization. In yet another experiment, this problem was avoided by the usage of a thermo-labile glucoamylase (Lancashire et al. 1989).

Simultaneous expression and secretion of glucoamylase and α-amylase activities has also been attempted, in both laboratory and industrial strains of *S. cerevisiae* (de Moraes et al. 1995; Shibuya et al. 1992; Steyn and Pretorius 1991). Some of the strains obtained were quite efficient in starch hydrolysis.

7.2 Flavor Components: Too Little and Too Much

The brewer and the brewing scientist along are regularly faced with the question: Which chemical components make up the beer flavor? Countless man-hours have been, and are still being, spent in breweries all over the globe to identify single flavor components, to find their taste thresholds and to define their contribution to the particular beer product. When relevant results are available the work can begin to find ways to increase or decrease the amounts of the particular flavor component in the final product. While many important compounds come from the barley and hops used, a few groups of yeast metabolites also take prominent roles in flavor formation.

One specific group of flavor constraints consists of thiols and sulfides. The impact of these compounds, bearing flavor descriptions as "rotten," "decaying," "onionlike" or "cooked vegetables," is usually considered to be negative, but some of these compounds take part in the larger synthesis of beer taste as such. One example of this is dimethylsulphide (DMS), the key compound in the smell of cooked sweet corn. While clearly objectionable at high concentrations, DMS at lower levels is believed to be a key component in the typical flavor of some lager brands. Dimethylsulphide is often found in concentrations just around its flavor threshold, and is therefore of concern. While some DMS comes directly from the wort, the majority of DMS found in the finished product represents a brewing yeast metabolite (Anness et al. 1979; Leemans et al. 1993; Hansen 1999; Hansen et al. 2002), a result of dimethylsulphoxide (DMSO) reduction by the *MXR1*-encoded methionine sulphoxide reductase (Hansen 1999). Using the two-step deletion procedure, lager brewing yeasts with diminished or neutralized capability to reduce DMSO were recently fabricated. These yeasts were shown to produce beer with 50–80% less DMS content than beer from an unaltered strain, but otherwise normal (Hansen et al. 2002). Another compound of high importance is hydrogen sulfide, H_2S, both because of the unpleasant flavor it imparts, and because of the thiols believed to be derived from it, methane- and ethanethiol (Tezuka et al. 1992; Walker and Simpson 1993). In the yeast cell, H_2S is condensed to activated homoserine, a crucial reaction that ensures assimilation of inorganic sulfur into important organic sulfur compounds such as cysteine, methionine, and glutathione (Figure 1). Metabolically upstream, H_2S is the product of sulfite reduction, and in turn sulfite is derived from sulfate ions taken up from the surrounding environment, through two intermediates (Figure 1). A few targeted approaches to controlling H_2S production have been attempted, taking

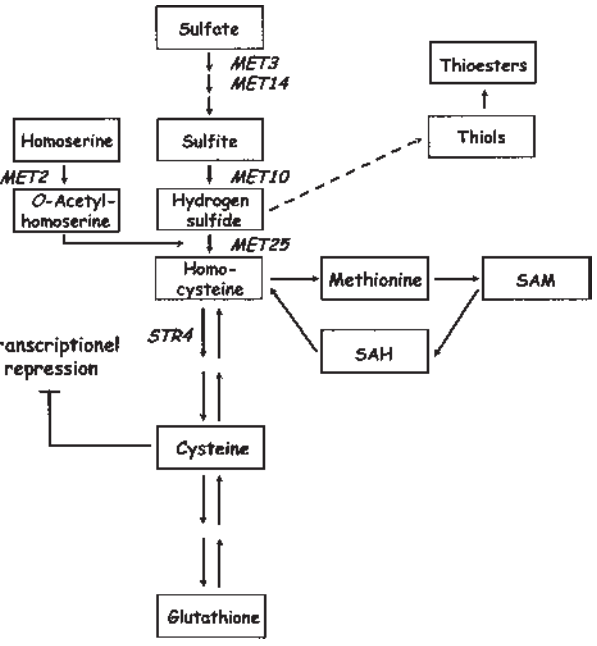

Figure 1 Sulfur assimilation and biosynthesis of sulfur-containing amino acids by *Saccharomyces* yeasts.

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advantage of the knowledge of the sulfur assimilation pathway. The product of the *STR4* (*CYS4*, *NSH5*) gene, cystathionine β-synthase, converts homocysteine formed by the H_2S-*O*-acetylhomoserine condensation into cystathionine. Tezuka et al. (1992) hypothesized that overexpression of *STR4* in brewer's yeast would lead to diversion of homocysteine, which would cause a higher activity of homocysteine synthetase and therefore a drop in H_2S accumulation. As expected, H_2S accumulation was suppressed. The reason for this, however, may not only be diversion of homocysteine but also an increase in the intracellular concentration of cysteine, a compound believed to be involved in repression of sulfur assimilation genes, including those leading to H_2S formation (Hansen and Johannesen 2000; Ono et al. 1996; Paszewski and Ono 1992). In a somewhat similar approach, Omura et al. (1995) found that an increase in the copy number of *MET25*, the gene encoding homocysteine synthetase (Figure 1), resulted in a 10-fold reduction in the amount of hydrogen sulfide in the growth medium. Finally, disruption in brewer's yeast of the *MET10* genes, encoding part of the sulfite reductase enzyme (Hansen et al. 1994), resulted in a dramatic increase in sulfite production and presumably an elimination of sulfide production (Hansen and Kielland-Brandt 1995; 1996b).

Another group of yeast metabolites of high importance for beer flavor consists of higher alcohols (the so-called fusel alcohols), and esters. Amino acids such as leucine, valine, and isoleucine may be formed *de novo* by brewing yeast but are also readily taken up by the cells if provided by the environment. The direct precursor for leucine biosynthesis is α-ketoisocaproate, but this compound is also the precursor for formation of isovaleraldehyde, which can be further converted to 3-methyl butan-1-ol, isoamyl alcohol, a compound that may impart a solventlike flavor to the beer. This fusel alcohol in turn is the precursor for isoamyl-acetate, an acetyl ester that imparts a strong banana and pear flavor to beer. As the amounts formed during fermentation are quite often at or above the flavor threshold level, immense efforts are being made to control formation of this compound. The genetics of leucine catabolism is complex (Hough et al. 1982), which is probably the reason for the very few published experiments with the genetic control of isoamylacetate formation.

In the pathway of leucine biosynthesis, *LEU4* encodes α-isopropylmalate synthase, the first step in the conversion of α-ketoisovalerate to α-ketoisocaproate. This enzyme is normally feedback-inhibited by leucine, but mutant yeasts resistant towards the toxic leucine analogue 5,5,5-trifluoro-DL-leucine (TFL) were found to contain leucine-insensitive versions of the enzyme (Satayanarayana et al. 1968). Comparable TFL-resistant mutants of *sake* yeast had increased production of isoamyl alcohol and its acetate ester (Ashida et al. 1987). Overexpression of *LEU4* from multiple, chromosomally integrated copies in an *S. cerevisiae sake* yeast caused TFL-resistance (Hirata and Hiroi 1991) and led to a higher production of isoamyl alcohol (Hirata et al. 1992). With the same strategy, mutants of lager brewing yeast with high production of isoamyl alcohol and its acetate ester have been obtained (Lee et al. 1995).

On the other hand, α-ketoisocaproate can also be formed by transamination of leucine taken up by the yeast. Kodama et al. (2001) studied the impact of overexpression of the leucine transporter *BAP2*, and found a positive influence on isoamyl alcohol production. Biosynthesis of isoamyl acetate is the result of acetylation of isoamyl alcohol. There are two alcohol acetyltransferase genes in *Saccharomyces* yeasts, *ATF1* and *ATF2* (Fujii et al. 1994; Nagasawa et al. 1998), and their corresponding enzymes account for most of the isoamyl acetate formed as well as other acetate esters. Thus, by overexpression of *ATF1* in *S. cerevisiae*, increased formation of isoamyl acetate was obtained, and after disruption of the gene production of isoamyl acetate was reduced by 80% (Fujii et al. 1996).

It may be possible to control the level of esters through the control of esterase activity. Watanabe et al. (1993) obtained mutants of *sake* yeast resistant towards isoamyl mono-fluoroacetate. These cells had an 85% reduction in esterase activity and a concomitant 1.5–2.0 fold increase in formation of isoamyl and isobutyl acetate. Manipulating the isoamyl acetate esterase gene *IAH1* directly in a *sake* yeast, Fukuda et al. (1998) found that the copy number of this gene had impact on isoamyl acetate production during sake brewing, even though the effect of varying the *ATF1* copy number was larger.

7.3 A Technical Problem: Beer Filtration

β-Glucan is a cellulosic compound containing β-1,3- and β-1,4 linkages, and forms a major part of barley cell walls. As a natural part of the germination process, a barley (1-3,1-4)-endo-β-glucanase is synthesized and present in the seed kernels, and this enzyme degrades much of the cell wall β-glucan during malting. However, depending on conditions of growth, harvest, storage, and malting of the barley, the amount of β-glucanase may be insufficient for complete degradation to take place. Moreover, during kilning and mashing of the malt, high temperatures inactivate the enzyme, meaning that hardly any is present in the wort to complete the cell wall degradation. As a result β-glucans are sometimes carried over to the wort and beer, which may obstruct the separation of spent grain from the wort, reduce filterability of the wort, and cause the formation of hazes and gels in the final product. The problem may be solved in the brewery by the addition of microbial β-glucanase at different stages of the brewing process, but as genes for β-glucanases were cloned and characterized from bacteria and fungi, it was obvious to try to express these genes in yeast. As it will be seen later, these experiments represent a quite successful branch of brewing yeast biotechnology.

Three different β-glucanase genes have been expressed in brewing yeast, namely those from *Bacillus subtilis*, barley, and the filamentous fungus *Trichoderma reesei*. While expression of the *B. subtilis* endo-β-(1-3,1-4)-glucanase

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Table 2 Biotechnological approaches to reduce diacetyl production during the main beer fermentation

Strategy	Gene	Methodology	Improvements/Comments	Reference
Disrupt α-AL formation	*S. cerevisiae ILV2*	*ILV2* antisense expression.	Reduced α-AL synthase activity but the transformant was compromised in growth and fermentation abilities.	Vakeria et al. 1991
Increase α-AL usage	*S. cerevisiae ILV5*	Extra few copies of native *ILV5* gene integrated at the *ILV2* locus.	Diacetyl production reduced by 35–43%, meaning that sub-taste threshold levels were not reached by the end of the main fermentation.	Goossens et al. 1993
Increase α-AL usage	*S. cerevisiae ILV5*	High copy number overexpression of native *ILV5* on 2 μm plasmids.	Diacetyl production reduced by 60%, meaning that sub-taste threshold levels were reached by the end of the main fermentation.	Villanueba et al. 1990
Increase α-AL usage	*S. cerevisiae ILV5*	Overexpression of *ILV5* by integration of two extra native *ILV5* copies at the *ILV5* locus.	Diacetyl production was significantly decreased. The asset of this strategy was that no foreign DNA was present after integration of the *ILV5* copies.	Mithieux and Weiss 1995
Diversion of α-AL	*Enterobacter aerogenes* α-ALDC	Yeast *ADH1*-controlled expression of bacterial gene on 2 μm plasmid.	Diacetyl production was decreased by 86%. The fermentation properties of the yeasts were otherwise unaltered.	Sone et al. 1987; Shimizu et al. 1989
Diversion of α-AL	*E. aerogenes* α-ALDC	Multiple integration at rDNA loci of yeast *ADH1*-controlled bacterial gene.	Insertion of more than 20 copies of the α-ALDC expression cassette resulted in a decrease in diacetyl production of 51–66%.	Fujii et al. 1990
Diversion of α-AL	E. aerogenes *and* Klebsiellαa terrigena α-*ALDC*	Yeast *ADH1*- or *PGK1*-controlled expression of bacterial genes on 2 μm plasmid.	With some of the transformant strains diacetyl concentration in the fermenting beer never reached the taste threshold level, and hence maturation was unnecessary.	Suihko et al. 1990
Diversion of α-AL	*E. aerogenes* and *K. terrigena* α-ALDC	Stable single-copy integration at *ADH1* or *PGK1* loci of *ADH1*- or *PGK1*-controlled bacterial genes.	*PGK1*-directed expression of α-ALDC resulted in a diacetyl concentration in the fermenting beer that never reached the taste threshold level. Hence maturation was unnecessary.	Blomqvist et al. 1991
Diversion of α-AL	*E. aerogenes and K. terrigena α-ALDC*	Integration of bacterial genes controlled by a modified yeast *ADH1* promoter.	Diacetyl concentration in the fermenting beer was reduced to the sub-taste threshold level, and hence maturation was unnecessary.	Onnela et al. 1996
Diversion of α-AL	Acetobacter aceti ssp. *Xylinum* α-*ALDC*	Yeast *PGK1*-controlled expression of bacterial genes. Integrated at rDNA loci or present on 2 μm plasmids.	Very little diacetyl produced in small-scale fermentation experiments. In pilot fermentation trials, diacetyl production was decreased by more than 70 %, and the necessary maturation time was shortened. The beer was otherwise unaltered.	Yamano et al. 1994a,b; 1995; Tada et al. 1995

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from the *S. cerevisiae ADH1* promoter was obtained in both ale and lager brewing yeasts (2 μm-based plasmid), very little enzyme was actually secreted (Cantwell et al. 1985). In a comparable experiment, but using the *S. cerevisiae* α-factor gene promoter and signal sequence, Lancashire and Wilde (1987) obtained good secretion of β-glucanase from a brewing yeast, which resulted in a reduction of the β-glucan content of the wort by 33% during an otherwise normal and satisfying beer fermentation process.

The heat-labile barley endo-β-(1-3,1-4)-glucanase has a low pH optimum, 4.7 as opposed to 6.7 for the *Bacillus* enzyme. As the pH of wort is around 5, the barley enzyme may be the most suitable choice. The barley β-glucanase was expressed from the yeast *ADH1* and *PGK1* promoters, and using the mouse β-amylase signal sequence, the enzyme was efficiently secreted from *S. cerevisiae* (Jackson et al. 1986; Olsen and Thomsen 1989).

The *Trichoderma reesei* endo-β-(1-4)-glucanase also has a relatively low pH optimum, and quite a lot of research into expression of the corresponding gene in brewing yeasts has been performed. Removal of most of the wort β-glucan was obtained after expression of the *T. reesei EG1* gene by the *S. cerevisiae PGK1* promoter, both when the hybrid construct was present on a 2 μm plasmid, and when it was integrated at a low copy number (2–5) (Enari et al. 1987; Penttilä et al. 1987a,b). However, these transformant yeasts showed a decreased fermentation rate (Penttilä et al. 1987b). In a continuation of these experiments, single copies of *EG1* expression cassettes (*EG1* fused to either the *ADH1* or *PGK1* promoters) were stably integrated in brewer's yeast at the *ADH1*, *PGK1* or *LEU2* loci. The expression cassette with the *PGK1* promoter inserted into the *PGK1* locus conferred a very high and adequate glucanolytic activity to the yeast (Suihko et al. 1991), and the fermentation properties of this yeast were satisfactory in every aspect studied. In addition, no bacterial sequences were present at the site of integration. In conclusion, this glucanolytic yeast represents a very advanced biotechnology-based brewing yeast.

7.4 Beer Maturation: How to Speed Things Up

The major rationale for the so-called secondary fermentation, or maturation period, is to coax the brewing yeast into removing the oxidation product of one of its own previously excreted metabolites, namely diacetyl. This compound imparts a, butterlike flavor, which is unwanted in lager beer. Diacetyl is the result of a nonenzymatic reduction of α-acetolactate (α-AL) that takes place in the fermenting beer. Even though the conversion to diacetyl is slow, a low-taste threshold necessitates removal of most of the formed diacetyl. To understand the different approaches to speed up this process, and thereby decrease the expensive maturation period, we must consider the formation of α-AL and the degradation of diacetyl by yeast. α-Acetolactate is an intermediate in the valine biosynthetic pathway, and the result of the *ILV2*-encoded acetohydroxy acid synthase conversion of pyruvate. The α-AL that does not leak out of the yeast cell is converted to α,β-dihydroxy-isovalerate by the action of the *ILV5*-encoded reductoisomerase. In two further enzymatic steps, the first of which is encoded by *ILV3*, the amino acid valine is finally synthesized. Diacetyl that has been taken up by the yeast cell is enzymatically converted to acetoin and then to 2,3-butanediol. The strategies to decrease the required maturation time have concentrated on two yeast-based approaches, namely a decrease in the speed of α-AL formation (*ILV2*) or an increase in the speed of its removal (*ILV5*), and a heterologous approach that is introduction of a bacterial enzymatic activity (α-AL decarboxylase, α-ALDC) for the direct conversion of α-AL into acetoin. Table 2 summarizes the results of more than 35 years of research on dealing with the diacetyl problem in brewing. Some of the solutions presented are very efficient and appear to be quite advanced.

Along the lines of strategy number one, Gjermansen et al. (1988) described an approach to the complete removal of acetohydroxy acid synthase activity in brewers' yeast, by the use of *in vitro* constructed *ilv2* deletions. It turned out, however, that *S. carlsbergensis* has a relatively low potential for valine uptake and thus a complete inactivation of Ilv2p activity is not practical (C. Gjermansen, unpublished, Kielland-Brandt et al. 1990). Some *S. cerevisiae* mutants resistant to the herbicide sulfometuron methyl (SM) are dominant alleles of *ILV2* (Falco and Dumas 1985) and, based on this observation, C. Gjermansen (unpublished, see Kielland-Brandt et al. 1989) devised an approach to obtain partially or fully blocked *ilv2* mutants in lager brewing yeast. This approach relied exclusively on *in vivo* recombination and mutation steps: Isolation of spontaneous SM-resistant mutants of allodiploid meiotic lager yeast progeny, UV mutagenesis, isolation of SM-sensitive pseudo-revertants, which are candidates for having only one remaining functional *ILV2* gene left, repetition of mutagenesis, and isolation of colonies with partial or full requirement for isoleucine and valine. The resulting allodiploid mutants are eventually mated to constitute an allotetraploid brewing yeast. Characteristics of strains constructed this way suggest that the method will be able to yield useful production strains. Although a quite elegant strategy to reduce *ILV2* expression by *ILV2* antisense expression resulted in a decrease in α-AL synthase activity, the transformant yeast was compromised in its fermentation performance (Vakeria et al. 1991).

The second yeast-based strategy to reduce diacetyl production relies on increased conversion of α-AL into α,β-dihydroxy isovalerate by increasing the amount of the responsible enzyme, encoded by *ILV5* (Gjermansen et al. 1988). Employing high copy plasmid expression or genomic integration of native *ILV5* genes in brewing yeast, an up to 60% reduction in diacetyl production was accomplished (Goossens et al. 1993; Mithieux and Weiss 1995; Villanueba et al. 1990). Hence, overexpression of *ILV5* can accomplish a reduction of diacetyl large enough to avoid maturation of the beer.

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Introduction of bacterial α-ALDC genes into brewing yeasts is the strategy that has been most elaborated upon. Initially, overexpression of α-ALDC genes from high copy number (2 μm) plasmids was carried out, and employing yeast *ADH1* promoter-directed expression of the *Enterobacter aerogenes* α-ALDC gene, an 86% decrease in diacetyl production was observed (Shimizu et al. 1989; Sone et al. 1987). In a comparable but somewhat more elaborate experiment *E. aerogenes* and *Klebsiella terrigena* α-ALDC expression was directed from either the *ADH1* or the *PGK1* promoter (Suihko et al. 1990). In pilot scale experiments with some of the constructed yeast strains the need for lagering could be eliminated. Single copies of the same types of constructs were stably integrated into one of the copies of *PGK1* or *ADH1* present in polyploid brewer's yeast (Blomqvist et al. 1991), and some of the yeast strains produced negligible amounts of diacetyl, rendering lagering unnecessary. No adverse effects on beer quality were seen. Fujii et al. (1990) integrated the *E. aerogenes* α-ALDC gene into brewer's yeast through cloned, homologous ribosomal DNA segments, taking advantage of the high copy number at the *RDN1* locus. More than 20 copies of the α-ALDC gene were thus inserted into the genome of the yeast, and unnecessary bacterial DNA sequences were lost through secondary recombination events. The α-ALDC activity was, to some extent, correlated to copy number, and testing at pilot scale showed a significant decrease in diacetyl content with some of the strains. For food approval purposes, the α-ALDC gene from the food-grade organism *Acetobacter aceti* ssp. *xylinum* has also been tested (Tada et al. 1995; Yamano et al. 1994a,b; 1995). Employing the constructed brewing yeast strains in pilot fermentation experiments, diacetyl production was reduced by more than 70%, and satisfactory beer could be produced at a significantly shortened time.

In conclusion, it can be said that from the technological point of view reduction of the maturation time can easily be achieved. For example, stably integrated single copies of the necessary genes can transform the brewing yeast to a strain that renders secondary fermentation unnecessary, without altering any other fermentation properties.

7.5 Flavor Stability: A Way to Increased Shelf Life

Beer is a very sensitive beverage: it needs to be kept in a dark and cool place to keep its taste. One of the worst aging-related off-flavors is t2n (*trans*-2-nonenal), which appears after prolonged storage of some beers at, for example, room temperature. Above its very low taste threshold (in the nanogram/liter range) this aldehyde imparts an undesirable cardboard or paperylike chemical taste and smell to the beer. A major problem in the brewing industry is therefore to keep the beer cool at all times, something that is quite costly and in hot climates very difficult. Fortunately, nature has provided the brewing yeast with the ability to dampen the destructive power of the barley-derived t2n, as this compound is inactivated by adduct formation to sulfite. As a natural part of its sulfur assimilation (Figure 1), sulfite is produced, and some is excreted to the ferment. The amount of sulfite produced depends on many factors, biotic as well as abiotic: the yeast strain or isolate used, the wort constitution, aeration of the wort, dimensions of the fermentation vessel, etc. Thus, the amounts of yeast-produced sulfite are not always adequate for flavor stabilization, and for this reason some efforts have been put into construction of lager brewing yeasts with increased sulfite production.

The strategies have been of two types: increase in the cellular metabolic flux from sulfate to sulfite and abolishment of the yeasts ability to turn sulfite into sulfide. Korch et al. (1991) tried to overexpress the *MET3* and *MET14* genes, encoding ATP sulfurylase and APS kinase, respectively, in *S. cerevisiae* and in brewer's yeast. The genes were expressed by their native promoters but with a highly increased copy number using 2 μm plasmids. A significant increase in sulfite production was seen, highest in strains deficient in sulfite reduction (*met5* mutants). Johannesen (1994) found an increase in sulfite production in a sulfite reductase deficient *S. cerevisiae* strain, after overexpression of an integrated *MET14* gene by the strong *TPI1* promoter. Recently this strategy was corroborated when concomitant overexpression of *MET14* and the gene for a sulfite efflux pump, *SSU1*, led to a 10-fold increase in sulfite secretion from an *S. cerevisiae* strain (Donalies and Stahl 2002). Along the lines of the second type of strategy, all four genomic copies of *MET10*, encoding part of the sulfite reductase enzyme, were inactivated in *S. carlsbergensis* brewer's yeast by partial deletion, using the two-step gene replacement procedure. This resulted in 7-fold or greater increase in sulfite production and elimination of sulfide production (Hansen and Kielland-Brandt 1995; 1996b). Disruption of the *MET2* gene in brewing yeast was also tried (Hansen and Kielland-Brandt 1995; 1996a). This work led to a general derepression of the sulfate assimilation due to a lack of *O*-acetyl homoserine (Figure 1), and therefore higher sulfite production. However, sulfide production was also increased in this yeast.

In spite of these seemingly successful metabolic engineering strategies, high production of sulfite by brewing yeast may not be advantageous at all. It was suggested (Dufour et al. 1989) that sulfite produced during fermentation could form nonmetabolizable adducts with certain carbonyl compounds and actually hinder removal of these. According to this model, sulfite added at the point of bottling might be more beneficial to the beer than yeast-derived sulfite. Recently inactivation of all four copies of *MET14* in lager brewing yeast was accomplished (Johannesen et al. 1999). Fermentation experiments with this yeast showed that sulfite produced by yeast during the main fermentation might actually result in a higher beer content of acetaldehyde (an off-flavor at high concentrations) and a longer lagering period due to higher diacetyl production. Furthermore this research showed that sulfite added at bottling was as efficient as yeast-derived sulfite in protection against t2n, and thus a yeast without any sulfite production at all may constitute a good

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solution, at least in countries where addition of sulfite to beer is legal.

8 CONCLUSIONS

For millenia, the process of beer making has employed yeast to turn carbohydrates into alcohol and CO_2. While a diverse array of yeast species have been and are being used for fermented beverage production, ascomycetes of the genus *Saccharomyces* are by far the organisms of choice. The key to the evolutionary appearance of such yeasts, the hallmarks of which are the ability to grow anaerobically and to produce ethanol even in the presence of oxygen, may have been an ancient whole-genome duplication event, followed by functional speciation of initially redundant gene copies. While at the beginning brewing yeasts have probably been selected simply by genetic adaptation to human-defined fermentation conditions, large efforts have gone into perfection of the performance of lager brewing yeasts, either by classical or molecular biological methods. Thus, improvements in the areas of better utilization of wort carbohydrates, better filterability of the finished beer, flavor stability, elimination of beer maturation, and control of certain flavor components or off-flavors, have been obtained by a range of methods.

The recent developments in what we could call the global analyses of living organisms have been quite stunning. By now we know the exact composition of all genes in *S. cerevisiae*, and this information is increasingly being used to gain knowledge on gene expression and on the accompanying fluxes of metabolites inside the yeast cell, and the associated area of beer fermentation. Current developments obviously add to the knowledge basis on which future breeding strategies can be built. So, the question is not whether the breeder can help the brewer improving the brewing yeast, but rather whether and when the consumers will be ready to accept beverages made by genetically modified organisms.

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Ethanol-Tolerance and Production by Yeast

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1 INTRODUCTION

Ethanol is an ideal fuel and the basis for countless chemical transformations. Worldwide interest in the production of ethanol as a source of renewable energy oscillated throughout the twentieth century, due to low oil prices. In recent years, the use of ethanol has increased dramatically. Ethanol attracted more attention due to its application as an octane enhancer and as a substitute for petrol. A significant number of automobiles in countries with vast supplies of fermentable sugar, such as Brazil, are nowadays fueled by ethanol or ethanol–petroleum mixtures (Ingledew 1993; Walker 1998). Continued increase of ethanol production will depend on process improvements, and reduction in fermentative ethanol costs. In those carbohydrate-to-ethanol processes where microorganisms are involved, optimal conversion requires cells that are tolerant to high concentrations of both substrate and product and are able to efficiently produce ethanol (Walker 1998). *Saccharomyces* yeasts are the most ethanol-tolerant of eucaryotic organisms, able to produce over 20% ethanol (Casey and Ingledew 1986). However, there are different ways of improving ethanol production: increasing the range of substrates used as feedstock, improving the efficiency of substrate conversion to ethanol, raising the fermentation temperature, or improving tolerances to ethanol and osmotic pressure. For these reasons attention has been given to yeasts other than *Saccharomyces* capable of fermenting substrates not accessible to the former such as inulin, starch, lactose, cellobiose, cellulose, hemicellulose, or xylose (Ingledew 1993; Walker 1998).

Ethanol, the main end product of anaerobic glycolysis in *Saccharomyces*, inhibits sugar fermentation and causes other unfavorable effects in yeast cells. For example, it is a noncompetitive inhibitor of growth rate and inhibits the transport of sugar and amino acids, and other processes associated with membrane lipids (Ingram and Buttke 1984; van Uden 1989; Walker 1998). Lipid composition of the plasma membrane is very important for ethanol-tolerance, consistent with structural changes observed in the cell membrane of microorganisms tolerant to high concentrations of ethanol (Ingram and Buttke 1984). Ethanol-tolerance also depends on environmental and nutritional conditions (Ingledew 1993). However, under fixed conditions non-isogenic strains differ in their ability to tolerate ethanol, and tolerance is a reproducible characteristic implying that it is genetically controlled (Jiménez and Benítez 1987; 1988). Genetic analysis has confirmed that the character is polygenic, and that the genes responsible for ethanol-tolerance are different in different strains (Jiménez and Benítez 1986). For this reason, hybridization has generated yeasts more tolerant to ethanol than their parentals. Current views of the genetic basis of ethanol-tolerance and production in yeast are summarized in this chapter. The relationship between nuclear and mitochondrial genomes and ethanol-tolerance is described, and recent developments in the genetic improvement of ethanol producing yeasts are also discussed.

2 YEAST CHARACTERIZATION FOR ETHANOL PRODUCTION

2.1 Physiological Characterization

2.1.1 Substrates of Potential Use for Ethanol Production

Suitability of any particular substrate for ethanol production is dictated mostly by feedstock availability, concentration of carbohydrate in the feedstock, and easy and rapid microbial attack (Ingledew 1993). The greatest potential for increasing supplies of ethanol comes from plant biomass. Starch (from maize, barley, wheat, oats, rye, rice, potatoes, cassava, or

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grain sorghum), juices from bananas, dates, pineapples, surplus or overripe grapes, sugar from sugar beet, sugar cane, sweet sorghum, Jerusalem artichoke, brewery wastes, molasses, or cellulosics can be used to make ethanol (Ingledew 1993; Walker 1998). Starch must be split by amylases into glucose and maltose. The fermentation of Jerusalem artichoke requires inulase-producing strains of *Kluyveromyces* (Rehm and Reed 1995). Molasses have been the most widespread raw material for ethanol production, although they are variable in quality and contain inhibitory compounds (Ingledew 1993). Liquid whey is available at low cost, but the use of lactose-utilizing yeasts is required (Table 1). However, the survival of the ethanol fermentation industry lies with the use of cellulosic wastes, even if starch-containing cereals are preferred nowadays in the manufacture of fuel ethanol (Ingledew 1993; Spencer and Spencer 1997).

Saccharomyces do not have the genes for amylases, cellulases, or β-galactosidases, or for degrading pentoses (Ingledew 1993). Several fermenting yeasts, including recombinant strains (see later) have been evaluated in converting lignocellulosic hydrolisates to ethanol. *Candida shehatae* and *Pichia stipitis* were the most successful, whereas *Kluyveromyces marxianus* was also effective in cellulose-to-ethanol conversions. Other important factors to select a microbial culture, in addition to ability to utilize a wide range of carbohydrates, include absence of metabolites other than ethanol, low pH optimum and high optimal temperature and resistance to several physicochemical stresses, rapid growth and fermentation rate, and high osmotolerance and ethanol-tolerance (Spencer and Spencer 1997).

2.1.2 Regulation of Substrate Utilization and Ethanol Production

a. Inhibition of Growth and Fermentation by Substrate and Ethanol. The production of high concentrations of ethanol is frequently limited by the inhibitory effect on productivity of the fermenting microorganism exerted by the substrate, the concentration of which affects osmotic pressure (van Uden 1989). Musts with lower sugar concentrations start to ferment sooner, and the sugar is completely fermented. High sugar concentrations inhibit the fermentation by their high osmotic pressure, which draws water from the yeast cells (Rehm and Reed 1995). Direct substrate inhibition of fermentative ability becomes significant somewhere between 15 and 25% sugar concentration (van Uden 1989). Values of specific ethanol production rate and specific sugar uptake decrease almost linearly with the increase in sugar concentration (Margaritis and Bajpai 1982) and the nature of the sugar is also important. In *Candida pseudotropicalis* the empirical constant of ethanol inhibition is different for glucose or lactose as substrates (Moulin et al. 1980). Glucose gave the lowest yield of ethanol production by *K. fragilis*, whereas galactose gave the highest, and lactose produced intermediate yields (Duvnjak et al. 1987). The inhibiting effect of sugar can be avoided by adding it in small amounts in staggered intervals. When the substrate is introduced in several batches ethanol yields are higher (Casey and Ingledew 1986; D'Amore and Stewart 1987) and cell viability is close to 95% compared to 40% for a single batch run (Casey and Ingledew 1986). In addition, strains differ in their

Table 1 Yeasts capable of producing ethanol from different carbohydrates

Glucose + sucrose	Lactose	Starch	Inulin
S. cerevisiae	*C. pseudotropicalis*	*S. diastaticus*	*C. pseudotropicalis*
S. ellipsoideus	*T. cremoris*	*K. marxianus*	*K. fragilis*
S. carlsbergensis	*K. fragilis*	*C. tropicalis*	*K. marxianus*
S. oviformis	*Brettanomyces* spp.	*C. shehatae*	*Torulopsis* spp.
S. pombe	*Torulopsis* spp.	*S. occidentalis*	*S. occidentalis*
	Debaryomyces spp.	*S. alluvius*	*S. castelli*
		S. castelli	*C. macedoniensis*
		E. fibuligera	*C. kefyr*
		E. castellii	*C. membranaefaciens*
Xilose	Cellobiose	Cellodextrins	*K. thermotolerans*
K. marxianus	*K. cellobiovorus*	*C. wickerhamii*	*S. fermentati*
C. tropicalis	*B. anomalus*		*S. cheresiensis*
C. shehatae	*C. lusitaniae*		*S. kluyveri*
K. cellobiovorus	*C. molischiana*		*S. malidevorans*
Clavispora spp.	*C. versatilis*		*Z. microellipsoides*
P. tannophilus	*C. wickerhamii*		*D. castellii*
P. stipitis			*T. delbreckii*
B. anomalus			*T. pretoriensis*
B. claussenii			*T. globosa*

Modified from Spencer and Spencer (1997).

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osmotolerance. Some *Saccharomyces* and *Candida* strains show increased growth rate inhibition by glucose concentrations over 10% (Jiménez and Benítez 1986; Moulin et al. 1980), whereas for flor yeasts this concentration rises to 25% (Jiménez and Benítez 1986). Efficient enhancement of ethanol production by stepwise addition of sugar depends, therefore, on the inhibitory kinetics by substrate (Duvnjak et al. 1987). In addition, some osmotolerant yeasts are also ethanol-tolerant but increasing production of glycerol for cell protection could represent a substantial loss of the ethanol produced, sometimes 40–60% of the carbohydrate consumed (Tubb 1984).

Yeast growth is also inhibited when the alcohol concentration of the fermenting must reach about 4%. Ethanol inhibition is the principal factor restricting fermentation rate and the concentration of ethanol obtained in ethanol production processes, therefore, the effect is of major economic significance (van Uden 1989). In addition, toxic metabolites such as acetaldehyde are formed within the cell (Rehm and Reed 1995). Growth and ethanol production rates are also inhibited by furfural, hydroxybenzoic, and acetic acid resulting from fermentation of lignocellulosic residues (Palmqvist et al. 1999). Some authors have also reported that high yield of ethanol can be limited by nutritional deficiencies rather than by ethanol toxicity (D'Amore and Stewart 1987). Higher levels of ethanol have been reached after nutrient supplements such as nitrogen or carbon sources, vitamins or magnesium (Dombek and Ingram 1986; Rosa et al. 1987). Addition of different supplements to molasses also increases ethanol production (Jiménez and Benítez 1986; Park and Sato 1982). The effect of adding each supplement is independent and additive in fermentation. However, findings that nutrient limitations are the cause of the cessation of growth in some fermentations (Ingledew 1993) do not detract from the importance of ethanol inhibition, since once the nutritional deficiencies are overcome, the role of ethanol inhibition is restored.

Concentrations of ethanol above 15% result in immediate inactivation of most organisms (Ingram and Buttke 1984). Among eukaryotes *Saccharomyces* is the most ethanol-tolerant organism able to produce ethanol up to 25% (v/v) (Ingram and Buttke 1984). Growth and fermentation are partly-linked parameters, so that yeasts continue to produce ethanol after growth has stopped, but an increase in ethanol-tolerant growth gives rise to a proportional increase in the rate of ethanol production (Jiménez and Benítez 1988). There is also a correlation between survival rate in aqueous ethanol and the levels of ethanol-tolerance in growth medium (Day et al. 1975).

Although cells stop fermenting at higher concentrations than that which completely inhibits growth, there are no striking differences between the response of growth and ethanol fermentation. The growth rate, μ is expressed as a function of the limiting substrate concentration s so that $\mu = \mu_0(s/K_s + s)$, where μ_0 and K_s are the maximum specific growth rate and the Monod constant, respectively (Luong 1985). During ethanol fermentation, the presence of ethanol decreases the specific growth rate. Ethanol inhibition affects μ but not K_s, therefore, growth rate is inhibited by ethanol in a noncompetitive manner (van Uden 1989) and can be represented as $\mu = \mu_i(s/K_s + s)$, where μ_i is the maximum specific growth rate in the presence of ethanol.

In addition, four types of dependence of μ on ethanol concentration (p) are reported in the literature: a linear relationship ($\mu = \mu_0(1 - p/p_m)$) derived for bacteria (Holzberg et al. 1967), an exponential relationship ($\mu = \mu_0 e^{-kp}$) for *Saccharomyces cerevisiae* respiratory-deficient mutants (Aiba et al. 1968; Bacila and Horii 1979), a hyperbolic relationship ($\mu = \mu_0(1/1 + (p/k))$) in *Saccharomyces* bakers' yeasts (Aiba et al. 1968). Exponential and hyperbolic relationships predict a continuous decrease of μ as p rises, but also imply that the cells are capable of growing and producing ethanol indefinitely. Other authors (Bazua and Wilke 1977) suggest the parabolic equation $\mu = \mu_0(1 - p/p_m)^{0.5}$ to describe the observed kinetics of product inhibition in *S. cerevisiae*. Other generalized nonlinear equations have also been proposed (Chattaway et al. 1988), and the differences in the reported experimental results attributed to the physiological conditions of the microorganisms and environmental conditions used (Luong 1985). Inhibition kinetics adjustable to linear relationships have been shown by laboratory and some industrial strains, parabolic inhibition by distillers', and linear relationship over 6% ethanol but no inhibition at lower concentrations in wine yeasts (Jiménez and Benítez 1986). In addition, the relationship of μ to ethanol varies exponentially for petite strains of *S. cerevisiae* and *S. sake* and grande strains of *S. cerevisiae* and *C. pseudotropicalis* grown under anaerobic conditions, but this relationship is linear in grande strains in aerobiosis (Aguilera and Benítez 1985; Holzberg et al. 1967; Moulin et al. 1980). The dependency of μ on ethanol varies according to whether or not the cells respire.

Most authors have studied the effect of ethanol concentration on growth and fermentation using added ethanol (van Uden 1989). However, it seems that ethanol inhibition of growth and fermentation occurs at lower concentrations of ethanol if the yeast produces it endogenously. This phenomenon has been described for laboratory, wine and brewing yeasts (Benítez et al. 1983; Day et al. 1975) and thought to be a result of its accumulating intracellularly to concentrations higher than those of the external medium (van Uden 1989). However, several authors (Guijarro and Lagunas 1984) demonstrate that the cell membrane are highly permeable to ethanol, that ethanol permeates the yeast cell plasma membrane without involvement of any carrier and the outflow rate of ethanol is greater than the ability of the organism to produce ethanol. Therefore, intracellular accumulation of ethanol is not feasible.

A possible explanation in the kinetic pattern when yeast produce their own ethanol as compared to added ethanol is that of an osmotic effect due to high substrate concentrations, since ethanol and substrate show synergistic effects (van Uden 1989). Growth kinetics of several yeast strains as affected by ethanol varies with the nature of the substrate and with the initial substrate concentration (Moulin et al. 1980).

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In the production of ethanol from sucrose, *S. cerevisiae* do not grow at 60% sucrose and growth is nonexponential over 40% (Benítez et al. 1983). Due to the synergistic effects of sucrose and ethanol, the concentration of 35% sucrose produces maximal amount of ethanol, above this concentration the rate of ethanol production and the final concentration decreases and is 0 at 70% sucrose. Cell growth under increasing salt concentration is more resistant to ethanol-induced leakage of UV-absorbing substances, and this is an index of ethanol-tolerance (Sharma 1997; Sharma et al. 1996), which indicates an overlap between osmo- and ethanol-tolerance in these strains.

b. Effects of Ethanol on Cell Membranes and Transport Systems. Most of the reported physiological results imply that cell membranes are important determinants of alcohol resistance and, more precisely that lipid composition in yeasts is of great relevance for the improvement of ethanol-tolerance (Thomas et al. 1978). During growth in ethanol *S. cerevisiae* synthesize lipids enriched in C18:1 fatty acyl residues to compensate for a decrease in palmityl residues. An unusually elevated accumulation of ethyl esters in lipid extracts of yeast cells in the presence of ethanol has also been observed (Guerzoni et al. 1999). An increase in the proportion of ergosterol, unsaturated fatty acids, and phospholipid, as well as a decrease in the sterol/protein ratio seem to be responsible for tolerance of *S. cerevisiae* and *Kloeckera apiculata* strains with different ethanol-tolerance (Alexandre et al. 1994; Chi and Arneborg 1999). Supplementation with ergosterol is also partly responsible for suppression of hypo-osmotic sensitive phenotypes in *S. cerevisiae* mutants (Toh et al. 2001). This and the reports on supplementation with unsaturated fatty acids favoring alcohol tolerance led to the conclusion that the cell membrane is the main target for ethanol inhibition (Thomas et al. 1978), and it also implies that membrane fatty acyl residues are important determinants of ethanol resistance. Ingram and Buttke (1984) also observed that *Escherichia coli* cells change their fatty acid composition when grown in the presence of alcohols and suggested that the changes represent an adaptive alteration compensating for the direct interaction of alcohols with the membrane.

There is an exponential increase in yeast plasma membrane fluidity with ethanol concentration (van Uden 1989), and a threshold concentration before the fluidizing effect, which indicates that cells exhibiting higher fluidity are expressing poorer membrane state rather than a mechanism of adaptation to ethanol. In fact, these cells do not improve their growth or fermentation rates. In contrast, *K. lactis* reduces the fluidity of its lipids by decreasing the unsaturation index, and a direct correlation between nonlethal ethanol concentration and unsaturation index was observed (Heipieper et al. 2000). Increased sensitivity of C18:3-enriched cells to heat and ethanol has been recently reported that is attributable to membrane damage associated with increases in membrane fluidity and oxygen-derived free radical attack of membrane lipids (Swan and Watson 1999).

By means of the extracellular acidification technique (Jiménez and van Uden 1985) it was found that the ethanol-tolerance of cell plasma membranes of highly ethanol-tolerant strains was very different from that of ethanol-sensitive strains. The increase in sensitivity was associated with an increase in passive diffusion of protons through the cell membrane, whereas the changes in the membrane tolerance were associated to changes in the protein rather than the lipid composition (Jimenez and Benítez 1988). However, the transport exponential inhibition constant by different alcohols correlates with their lipid/buffer partition coefficients. This indicates that hydrophobic membrane sites are the primary targets of alcohol inhibition of transport (Leao and van Uden 1984). The effects of ethanol on the plasma membrane are attenuated by the presence of trehalose and urea (Figure 1). However, over certain ethanol concentrations urea enriches proteins with water and excludes ethanol, whereas trehalose increases protein–ethanol interactions leading to a stronger inactivation (Lopes and Sola-Penna 2001). The accumulation of trehalose is a critical determinant of stress resistance in *S. cerevisiae* and *Schizosaccharomyces pombe* to the point in yeasts where trehalase gene *ATH1* has been disrupted or trehalose-6-phosphate synthase has been overexpressed accumulate elevated levels of trehalose which correlate with increased tolerance to toxic levels of ethanol, dehydration, heat shock, osmotic stress, and freezing (Figure 1) (Kim et al. 1996; Soto et al. 1999).

Alcohols also inhibit transport systems in eukaryotic microorganisms. The effect of ethanol on glucose transport has been studied in *S. cerevisiae* using D-xylose as a nonmetabolizable sugar analog (Leao and van Uden 1984).

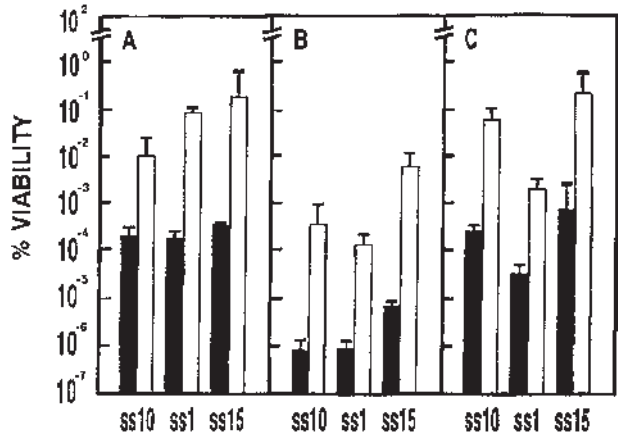

Figure 1 Colony survival of *S. pombe* SS10 (wild type), SS1 (Δ*tps1*), and SS15 (Δ*ntp1*) strains containing pREP3X (TPS1) after different stresses. Before each treatment cells were cultured in the presence (■) or absence (□) of thiamine. (A) Freeze-thawing, (B) dehydration, and (C) 15% exogenous ethanol shock. (TPS1, trehalose-6P synthase; NTP1, neutral trehalase) (Soto et al. 1999).

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Ethanol does not affect the affinity of the transport system for D-xylose (noncompetitive inhibition), but rather lowers the rate of sugar uptake. Similar results were found when glucose, fructose, and maltose transport were examined in strains of *S. cerevisiae* and *K. fragilis* (van Uden 1989). Ethanol also inhibits the transport of acetic acid in a noncompetitive way (Casal et al. 1998). Furthermore, when using a nonmetabolizable analog of ammonium, it was found that in *S. cerevisiae* ethanol has a more powerful effect on ammonium transport than on sugar transport (van Uden 1989). At ethanol concentrations at which the transport system for glucose and ammonium are inhibited, the inhibition of the latter was much stronger than that of the former, thus indicating that fermentation may still proceed at ethanol concentrations at which growth is already completely inhibited. In *Candida wickerhamii* grown in cellobiose, which is externally hydrolyzed, ethanol reversibly inhibits the two glucose transport systems, facilitated diffusion and glucose-proton transport with exponential noncompetitive kinetics (van Uden 1989). In addition, ethanol noncompetitively inhibits ATPase of *S. cerevisiae* plasma membrane (Cartwright et al. 1987).

c. Effects of Ethanol on Glycolytic Enzymes. Ethanol is a major end-product of glycolysis in *S. cerevisiae* and, therefore, the observed inhibition of growth and fermentation by ethanol may reflect its effect on enzymes involved in the glycolytic pathway. Noncompetitive reversible inhibition by ethanol has been reported of hexokinase, pyruvate decarboxylase, alcohol dehydrogenase, and α-glycerophosphate dehydrogenase (Figure 2) (Llorente and Sols 1969) and high levels of these enzymes were necessary for faster ethanol production. Strains of *S. cerevisiae* identified as fast or slow ethanol producers showed correlation with the levels of pyruvate decarboxylase, alcohol dehydrogenase, aldehyde dehydrogenase, and in some reports of invertase (Gokhale et al. 1987; Sharma et al. 1996). There is also a general resistance of glycolytic enzymes to ethanol denaturation in ethanol-tolerant strains at concentrations that inactivate the corresponding enzymes in sensitive strains (Llorente and Sols 1969). In addition, ethanol concentrations that denature yeast glycolytic enzymes (measured *in vitro*) and inhibit fermentation are higher than those that inhibit growth (Pascual et al. 1988; van Uden 1989). Comparative studies of ethanol effects on glycolytic enzymes, the glucose uptake system and proton extrusion rate have indicated that up to 2 M ethanol does not affect glucose uptake rate or glycolytic activities whereas transmembrane proton flux was the most ethanol-sensitive, representing the first target of ethanol action on fermentation (Pascual et al. 1988). To conclude, ethanol inhibition of glycolytic enzymes involved in fermentation of at least glucose, fructose, and sucrose does not appear to be a substantial problem to yeast, although phosphoglycerate kinase and pyruvate decarboxylase are partly inhibited by 10% ethanol (van Uden 1989). However, since these enzymes are in excess amount required for maximum flux it is unlikely that their inhibition by ethanol is a major cause of the declining fermentation rate as ethanol concentration increases.

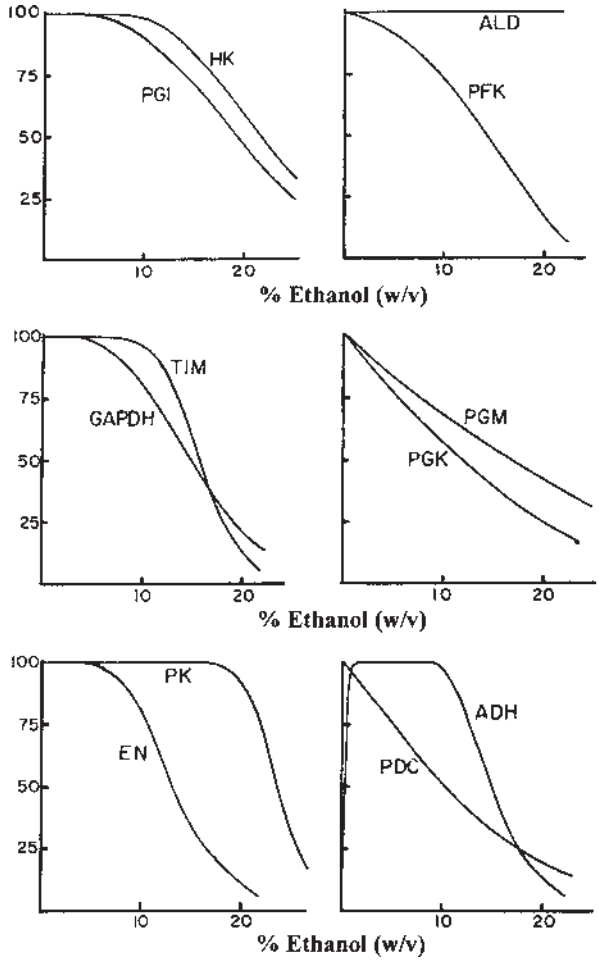

Figure 2 Inhibition curves for the effect of ethanol on the 12 yeast glycolytic enzymes. Enzyme name abbreviations: HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; ALD, fructose 1,6-biphosphate aldolase; TIM, triose phosphate isomerase; GAPDH, glyceraldehyde phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; EN, enolase; PK, pyruvate kinase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase (van Uden 1989).

d. Synergistic Effects of Ethanol, Temperature, and Other Stressing Agents. Synergy between inhibitory effects of ethanol and other agents, mainly temperature has long been studied. Rise in temperature has been observed to be accompanied by decrease in inability to tolerate ethanol (van Uden 1989), so that ethanol toxicity for *S. cerevisiae* and other yeasts may be enhanced by rises in the process temperature, leading to a depression of growth and fermentation and a loss of viability of the yeast cell

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population (van Uden 1989). It has been indicated earlier that when yeasts are grown in the presence of ethanol the lipid composition of their membranes changes, and this may represent an adaptive response. It has also been suggested that membrane lipid composition is a function of the specific growth rate (μ) as well. By using a chemostat, the two variables, μ and ethanol concentration were separated, and the effect of each on viability was studied. Figure 3 shows that cell populations grown at the same ethanol concentration but at different dilution rates possess increased resistance to thermal death, whereas cells grown at the same dilution rate and different ethanol concentrations did not differ in their thermal resistance. Results indicate that changes in membrane composition induced by ethanol do not increase thermal resistance (van Uden 1989), whereas changes due to diminishing μ do increase thermal resistance.

Heat and ethanol exposure induce essentially identical stress responses in yeast, and induce heat shock proteins (Hsp) (Piper 1995), some of which such as Hsp104, contribute to thermotolerance and ethanol-tolerance, while others are antioxidant enzymes. It is worth noticing that expression of Hsp104 is repressed during fermentation in brewery yeasts (Brosna et al. 2000). Temperature shifts from 23 to 36° induce the transient synthesis of a set of Hsp that confer protection against a subsequent and otherwise lethal rise in temperature (Hohmann and Mager 1997). Ethanol and other stressing agents, including glucose limitation also induce Hsp proteins, suggesting that these proteins function in a general cellular response to stress (Bataillé et al. 1991). Some of these proteins are involved in the metabolism of trehalose, so that enzyme modification, which produces increase in trehalose accumulation also results in increased tolerance to osmotic stress, high ethanol concentrations, thermotolerance, and increased tolerance to freezing and thawing (Figure 1) (Soto et al. 1999). Others are hydrophobic proteins that play essential roles in ethanol-tolerance. Sherry strains of *S. cerevisiae* form a biofilm on the surface of the wine when grape sugar is depleted at the end of fermentation (Martínez et al. 1995). Film formation takes place after the cells duplicate their hydrophobicity (Martínez et al. 1997a,b,c). This increase in hydrophobicity has been associated with hydrophobic proteins rather than lipids (Martínez et al. 1997c) and correlates with increased ethanol-tolerance, thermotolerance, and resistance to sonication (Castrejón 2000). Furthermore, a highly hydrophobic 49 kDa cell wall mannoprotein has been isolated from cells only during the film phase (Alexandre et al. 2000). Another protein, Hsp12 that protects liposome membranes against desiccating-induced leakage seems also to be involved in flor formation and ethanol-tolerance. Hsp12 is active in stationary phase after glucose depletion in the presence of ethanol (Zara et al. 2002). However, flor yeasts delete for Hsp12, whose product are unable to form film but do not become more temperature-or ethanol-sensitive.

During the velum phase, cells are subjected to hydric stress (velum forms on the surface of sherry wine), oxidative stress (sherry has no fermentable carbon and nitrogen sources), and stress induced by the high concentrations of ethanol and acetaldehyde of the sherry wine. With regards to the hydric stress, structural proteins called hydrophobins are involved in the emergence of microbial aerial hyphae, and lower the surface tension resulting in the hyphae to grow into the air. A single protein was isolated from the supernatant obtained after sonication and filtration of flor cells, and further application of

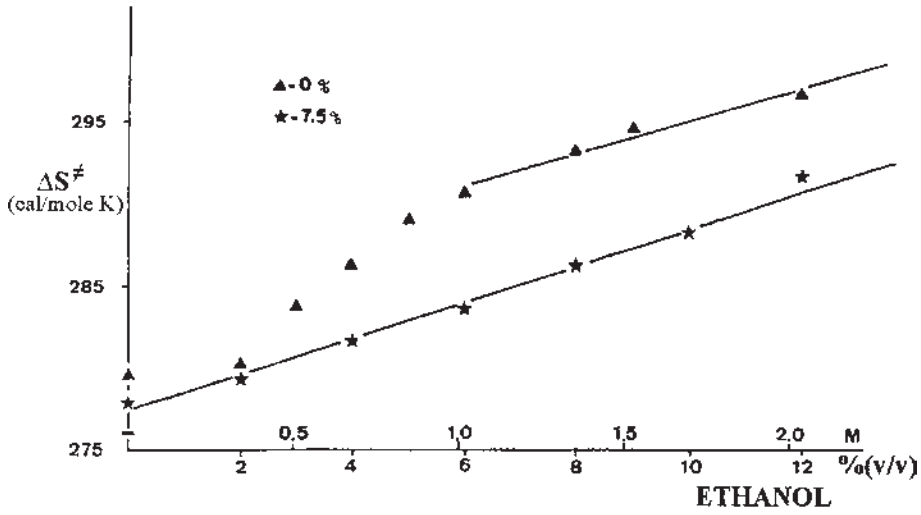

Figure 3 Dependence of $\Delta S^{\neq}$, the entropy of activation of thermal death in a strain of *S. cerevisiae* on the concentration of ethanol in the suspending medium in which the death experiments were performed. (Upper curve) Cells grown in the chemostat at dilution rate 0.09 h^{-1} without ethanol. (Lower curve) Cells grown in the chemostat at dilution rate 0.09 h^{-1} in the presence of 7.5% (v/v) ethanol. (van Uden 1989).

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procedures to purify fungal hydrophobins. This highly hydrophobic protein of 20.5 kDa has features similar to type II hydrophobins and possesses the capacity to aggregate flor cells (Castrejón 2000). Superoxide dismutase (Sod) is also induced in the presence of ethanol. Sod1p protects against the oxidative stress resulting from the metabolites originated during respiratory metabolism, and has been located in the cytosol. Yeast mutants defective in Sod1 are oxygen-sensitive, show an elevated rate of spontaneous mutations probably due to oxidative damage of the DNA, and are unable to grow in nonfermentable carbon sources.

e. Effects of Ethanol on Mitochondria. Yeasts have thermosensitive target sites which, if irreversibly destroyed, leads to loss of cell viability (van Uden 1989). Exponential death rates at supraoptimal temperatures concur with exponential petite mutation, suggesting that mitochondria are targets of thermal death in yeasts (van Uden 1989). Ethanol decreases the maximum temperature for growth (T_{max}) of *S. cerevisiae*. A similar enhancement of thermal death is observed when other alkanols are used, which correlates with their lipid-buffer partition coefficients and suggests that the thermal death sites are associated with membrane systems (van Uden 1989). The effects of ethanol on T_{max}, thermal death, and petite mutations suggest that the target for the three phenomena is located on a mitochondrial membrane (Jiménez and Benítez 1988).

It seems that nuclear genes are responsible for tolerance to ethanol at concentrations that allow cell growth. Under extreme conditions of high ethanol concentrations and/or temperatures the mitochondrial genome is solely responsible for ethanol-tolerance, since cells do not grow or divide, and the parameter that determines ethanol-tolerance is cell viability. Under extreme conditions of ethanol and temperature the specific death rate, μ_d is proportional to petite induction rate, λ (Figure 4). When mitochondria are transferred from highly ethanol-tolerant strains to laboratory and more ethanol-sensitive strains, μ_d and λ values in the heteroplasmonts are equal to μ_d and λ values shown by the ethanol-tolerant donor strain (Jiménez and Benítez 1987), which indicates that mitochondrial functions and survival are linked parameters and that the mitochondrial genome is responsible for cell survival under extreme conditions of high ethanol concentrations and/or high temperatures.

Flor yeast mitochondria are highly resistant to ethanol and maintain their functionality in the presence of this compound (Martínez et al. 1997b). At the same time, *S. cerevisiae* wine yeasts show a considerable amount of mtDNA variation (Esteve-Zarzoso et al. 2001; Martínez et al. 1995; Mesa et al. 2000). This is probably due to the mutagenicity of the high ethanol environment, since the spontaneous level of petite mutants increased 10-fold at 24% ethanol (Bandas and Zakharov 1980). Due to the fact that metabolism is oxidative in sherry wine, it was suggested that there was a high rate of mutation of mtDNA induced by ethanol, but the need of functional mitochondria discarded any change leading to nonfunctional genomes (Martínez et al. 1995). It has also

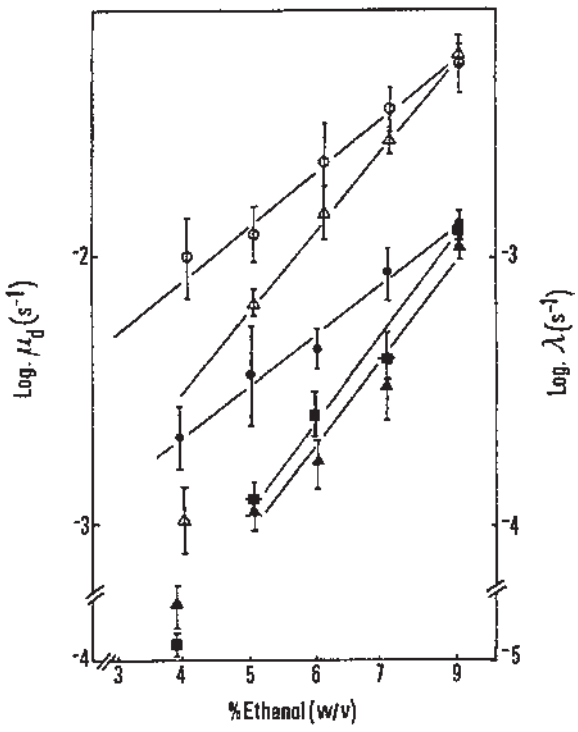

Figure 4 Death rate (μ_d) (●, ▲, ■) and the rate of rho-formation, λ, (○, △) of laboratory strain ABQ21 (●, ○), wine strain ACA21 (■), and heteroplasmon ABQ21-H1 (▲, △) grown in YPDE supplemented with the indicated ethanol concentrations at 38°C (Jiménez and Benítez 1988).

been reported (Ibeas and Jiménez 1997) that the mechanism for ethanol mutagenicity was mtDNA loss in flor yeasts, so that, rather than changes in the mtDNA sequence, the petite mutants analyzed lacked mtDNA completely. Ethanol is an active membrane solvent and its mutagenic effect was attributed to mitochondrial membrane alterations leading to mtDNA loss.

Reports on the effect of ethanol and acetaldehyde on yeast chromosomal DNA indicated that ethanol produces DNA breaks, but that acetaldehyde has a stronger deleterious effect on chromosomal DNA (Ristow et al. 1995). Wine yeasts should be expected to undergo continuous DNA changes due to errors induced during the mechanisms of DNA strand break repair via recombination. This would explain the high frequency of chromosome reorganizations and the divergence found between laboratory and wine yeast chromosomal organizations (Puig et al. 2000). Changes in mtDNA sequences can be the main (although indirect) effect of acetaldehyde or ethanol, which introduce breaks in both the nuclear DNA and mitochondrial. When DNA is repaired, mtDNA polymerase, which lacks proofreading capacity, introduces changes detected in RFLP pattern, whereas nuclear DNA is properly repaired. The preferential effect of

acetaldehyde and ethanol on mtDNA could, therefore, be partly the result of a better system to repair yeast nuclear DNA, and the lack of proofreading activity of the mtDNA polymerase. Mutagenic effect of ethanol and acetaldehyde strongly contribute to the high frequency of mtDNA variability in wine yeasts. Acetaldehyde, and to a lesser extent ethanol, induce petite mutants in *S. cerevisiae* yeast strains, and the mechanism accounting for this induction is an alteration of mtDNA sequence. Only after prolonged incubation, mtDNA loss finally occurs.

Ethanol toxicity has been correlated with the production of reactive oxygen species (ROS) in the mitochondria, and mitochondrial superoxide dismutase (MnSOD) has been reported as essential for ethanol-tolerance (Costa et al. 1993; 1997) and for protection against oxygen toxicity (van Loon et al. 1986; Piper 1999). Consistent with the generation of hydroxyl radical from superoxide, mtDNA may undergo dramatically elevated spontaneous mutagenesis, presumably due to oxidative damage to this mtDNA. Increased ethanol-tolerance of wine yeasts might be due to their ability to prevent superoxide radical-induced damages, and this is supported by the fact that a highly efficient MnSOD renders more ethanol-tolerant yeast cells (Costa et al. 1993; 1997).

Preliminary results indicate that at least SOD1 superoxide dismutase mRNA level is considerably higher in flor yeast strains than in laboratory strains. When flor yeasts are grown in sherry wine (Peñate et al. 2001), mRNA levels dramatically increase in velum phase as compared to stationary or exponential phase. Furthermore, at velum phase flor yeast cells become simultaneously highly ethanol- and thermo-tolerant (Castrejón 2000), and these phenomena is associated with a higher superoxide dismutase activity (Costa et al. 1993; 1997). A secondary effect of ethanol would also be chromosome loss, due to its role as membrane solvent; this would explain the high frequency of aneuploidies observed in wine yeasts (Guijo et al. 1997; Martínez et al. 1995).

2.2 Genetic Characterization

2.2.1 Nuclear Genome

It is speculated that more than 250 genes are involved in the control of ethanol-tolerance in yeast (Pretorius 2000). The first evidence of a polygenic character of ethanol-tolerance was that of crosses between haploid products of *S. cerevisiae* with different levels of tolerance which indicated that ethanol-tolerance in yeast species is controlled by a large number of genes (Ismail and Ali 1971). Diploids had a wide range of ethanol-tolerance, sometimes exceeding that of their parental, thus indicating complementation between nonisogenic strains. In addition, analysis of the meiotic products from a cross of two strains indicated that the progeny were as tolerant or more sensitive to ethanol than the parental, thus showing that ethanol sensitivity was due to recessive alleles (Jiménez and Benítez 1987). Analysis of the tolerance/sensitivity to ethanol of the parentals and of the meiotic products allows to estimate the *minimal number* of genes involved in the tolerance to ethanol of the more ethanol-tolerant parental with regards to the less ethanol-tolerant. There was a lack of correlation between genes which limit growth in the absence of ethanol, and those which do so at different ethanol concentrations, indicating that inhibition by ethanol is the result of the inhibition of different cellular functions with increasing ethanol concentrations (Jiménez and Benítez 1987).

The isolation of growth ethanol-sensitive mutants by mutagenesis with nitrosoguanidine provided further information on ethanol inhibition effects. Some mutants were temperature-sensitive and were affected in different genes, confirming that ethanol-tolerance is based on many genes, some affecting thermotolerance as well. The glycolytic pathway was not affected in the mutants, indicating the enzymes to be highly resistant to ethanol (Aguilera and Benítez 1985) and that growth is regulated by gene products not related to fermentation. This last point was confirmed by the isolation of mutants growth-sensitive to ethanol but which ferment like the wild type. Some ethanol-sensitive mutants that reduce the ability to synthesize ergosterol recovered their ethanol-tolerance after being transformed with *ERG6* gene involved in the ergosterol biosynthesis pathway, thus suggesting *ERG6* gene to play an important role in ethanol-tolerance (Inoue et al. 2000). Ethanol-sensitive mutants have also been obtained by transforming yeasts with a transposon (Takahashi and Shimoi 2001). Identification of the targets and further deletion of the corresponding genes produced ethanol-sensitive disruptants which also displayed sensitivity to lytic enzymes and antibiotics that act on the cells walls, thus indicating that the integrity of the cell wall is also important in ethanol-tolerance.

2.2.2 Mitochondrial Genome

Mitochondrial mutants with total inability to respire have a lower tolerance to ethanol than grande strains (Aguilera and Benítez 1985; Hutter and Oliver 1998). In addition, the frequency of petite mutants, either spontaneous or ethanol-induced is higher in laboratory strains than in ethanol-tolerant ones such as wine or distiller's yeasts (Jiménez and Benítez 1988). The greater stability in high ethanol concentrations of the grande phenotype conferred by the highly ethanol-tolerant yeast mitochondrial genome would be the main factor responsible for the greater viability displayed by yeast strains possessing these mitochondria because their grande phenotype permits respiration in conditions where other mitochondria are not functional.

An extensive study of wine yeast mitochondrial genomes indicated a high variability observed in gene order, connected with the presence of long intergenic regions containing recombinogenic sites. Gene order of *S. uvarum* suggested that the mitochondrial genome of the *S. cerevisiae*-like strains may have evolved from an ancestral molecule, similar to that of *S. uvarum* through specific genome rearrangement (Cardazzo et al. 1998). Furthermore, differences in gene order were due to translocations, confirming the relevant role

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of gene rearrangements in the evolution of yeast mtDNA genomes. In several of these wine strains, hybridization, as well as the compatibility nucleus–mitochondria have been confirmed by strains possessing the same RFLP of their mtDNA but different chromosomal patterns, and also the other way round. Successful restoration of respiration in *S. cerevisiae* petite mutants was achieved by transplacement of mitochondria isolated from *S. bayanus*, *S. capensis*, *S. delbrueckii*, *S. exiguus*, *S. italicus*, and *S. oviformis* (Osusky et al. 1997). It appears that in some species, most of the genetic information originally located in mitochondria was translocated to the nuclear compartment during evolution. A given functional mitochondrial gene can be either mitochondrially or nuclearly encoded, but in general nuclear information can change the structural organization of mitochondrial genome (Hartmann et al. 2000), and mutants affected in nuclear genes which regulate mitochondrial functions, such as those which release glucose repression and control repression of mitochondrial biogenesis have frequently been described (Brown et al. 1995). When mitochondria are transferred from wine to laboratory yeast strains they confer to the laboratory recipient strain a considerable increase in tolerance to ethanol, temperature, and mitochondrial loss induced by ethanol, when the mitochondrial and nuclear genomes are compatible (Jiménez and Benítez 1988).

3 IMPROVEMENT OF ETHANOL PRODUCTION

3.1 Improvement of the Environmental Conditions

Environmental factors such as yeast inoculum cell density, temperature, and key nutritional requirements play important roles in dictating yeast fermentation performance. For example, ethanol yields of over 21% in molasses and 23% wheat mashes have been achieved by optimization of magnesium ions and assimilable nitrogen availability (Walker 1998). Yeast growth is stimulated by magnesium whereas ethanol production is more efficient when both calcium and magnesium are added (Ciesarova et al. 1996). In addition, several fermentation strategies based on batch, fed-batch, and continuous processes have also been considered for ethanol production. Those systems which increase yeast cell densities such as immobilization or cell recycle (Jiménez and Benítez 1986) have led to higher ethanol productivities.

The development of processes for ethanol formation aimed at maximizing the yield of ethanol produced per amount of substrate (Verduyn et al. 1992). Product formation can be classified on the basis of its relation to the function of the organism. This is what happens when we refer to end products of energy metabolism such as ethanol (Pirt 1975). As metabolism is usually adjusted to produce the minimum amount of essential metabolites, the whole carbon source must be frequently converted into biomass and/or the end products of energy metabolism. Establishing the relationship between growth and product formation is necessary if rate and yield products are to be optimized. Ethanol productivity depends on whether ethanol formation is linked to growth (Andrews 1984). If both processes are totally linked, the amount of ethanol formed (p) is directly proportional to the biomass formed (x); if they are not linked, the specific rate of ethanol formation (q) is independent of the growth rate (μ); if q is partly linked to μ, the ethanol formed not linked to growth results from the maintenance energy requirement or maintenance coefficient (m) (Figure 5) (Benítez et al. 1998). The relationship between growth and ethanol productivity can be established by measuring q (Meyer et al. 1992) or product/biomass yield (Ypx) (Aguilera and Benítez 1989) (Figures 5

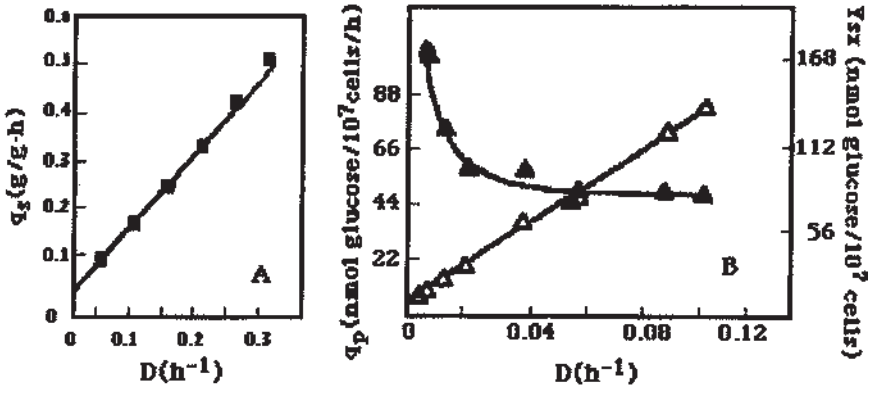

Figure 5 (A) Determination of the maintenance coefficient (m) for *C. blankii* in 50% dilute bagasse hemicellulose hydrolysate at pH 5.0 and 38°C by plotting the specific rate of substrate utilization (q_s) vs. dilution rate. The intercept ($m = 0.0303$ g/gh) was calculated by linear regression with a correlation coefficient of 0.9994 (Meyer et al. 1992). (B) Variation in the metabolic coefficient q_p (nmol glucose consumed/10^7 cells/h) (empty triangles) and the substrate/biomass yield, *Ysx* (nmol glucose consumed/10^7 cells) (black triangles) with the dilution rate, D (h^{-1}) in the rho-strains of *S. cerevisiae* grown in continuous culture with nitrogen-limiting substrate (Aguilera and Benítez 1988).

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and 6). Growth and ethanol production are partly linked parameters (Aguilera and Benítez 1989) and therefore *Ypx* increases at low growth rates. Ethanol production can be maintained when the cells are not growing, thus increasing the yield considerably. This partly linked relationship allowed the isolation of ethanol-sensitive mutants unable to grow at 0.5 M ethanol but capable of fermenting at ethanol concentrations over 1.5 M (Aguilera and Benítez 1985). In general, *Ypx* changes differently depending on whether a product is dependent on μ or not (Pirt 1975), and once this correlation is established the adjustment of μ to each particular situation can be carried out; processes aimed at biomass production have to be conducted at a very low growth rate, but on the other hand, processes in which ethanol is the desired product can be carried out without any limitations (Figures 5 and 6).

3.2 Genetic Improvement of Yeast for Ethanol Production

3.2.1 Genetic Features of Industrial Yeasts

Chromosomal polymorphism has been described to be higher in industrial yeasts than in laboratory strains. Among industrial yeasts, the literature concerning chromosomal polymorphism of brewers', bakers', or distillers' yeasts is very scarce, whereas that of wine yeasts is more abundant (Codón et al. 1997). Among wine strains, high chromosomal polymorphism has been reported. However, among them, film-forming strains do show scarce variability. It may be the case that the selective conditions which are so severe (lack of fermentable carbon sources and over 15% ethanol) have favored an almost unique chromosomal pattern, similar in all flor yeasts and different from those of other industrial or laboratory yeasts (Martínez et al. 1995). This lack of polymorphism may also be related to the scarce presence of Ty1 elements (although Ty2 are abundant in these strains).

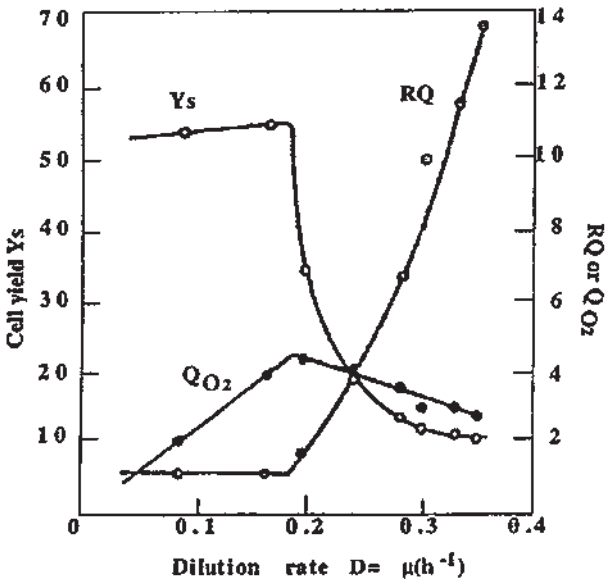

Figure 6 Effect of dilution rate on yeast metabolism in continuous culture. Y_s, yield in g solids per 100g of substrate; Q_{O2}, respiration rate in M/g yeast solids per h; RQ, respiratory quotient (Benítez et al. 1998).

In order to identify each chromosome specifically, they are hybridized with probes corresponding to genes which, in laboratory strains, are known to be present once in the genome. Chromosomes of industrial yeasts, including wine yeasts, hybridize in all cases with these probes, indicating that DNA similarity with these genes is very high. Hybridization confirms the existence of several homologous chromosomes of different sizes; interchromosomal (translocations) and intrachromosomal (deletions, duplications, and inversions) reorganizations have often been found as well (Codón et al. 1997).

If polymorphism of chromosomes reflects selection under specific conditions, extreme selective conditions such as the presence of high ethanol concentration or oxidative stress should favor any chromosomal reorganization, which results in the increase of viability or of growth rate under such conditions. In all flor strains examined by Guijo et al. (1997) polysomy of chromosome XIII was observed. This chromosome contains the *ADH2* and *ADH3* loci that encodes for the ADHII and ADHIII isoenzymes of alcohol dehydrogenase and which are involved in ethanol oxidative utilization during biological aging of wines. In bakers' strains grown in molasses whose main carbon source is sucrose, *SUC* gene, which encodes invertase has been amplified and translocated to several chromosomes as judged by the presence of several bands that hybridize with the probe, and a similar phenomenon seems to have occurred in distillers' yeasts (Codón et al. 1997). The wine yeasts analyzed, which in their natural environments ferment glucose and fructose but not sucrose, only possess a single band. In addition, the quality of brewing strains is largely determined by their flocculation properties, and several dominant, semidominant, and recessive flocculation (*FLO*) genes have been recognized in those brewing strains.

When compared with laboratory haploid strains, chromosomal patterns for enological *S. cerevisiae* strains were interpreted as some bands being homologous chromosomes of different sizes. This suggestion has been further confirmed by karyotype analysis of meiotic segregates of some strains (Puig et al. 2000). In addition, analysis of a number of independent crosses between haploid testers and unselected populations of spores of different wine strains allowed to distinguish between disomic, trisomic, or tetrasomic chromosomal complements in the parents, and showed that linkage relationships among the chromosomes of the wine strains were not identical to those of the laboratory testers. Translocations seemed to have placed genes found on specific chromosomes of laboratory strains on different chromosomes present in different copy numbers in wine

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Table 2 Ploidy (*n*) of different *S. cerevisiae* industrial strains

Strains		Ploidy (*n*)
A		
YNN295	G	1
DS81	G	2
DADI	B	1.5
VS	B	2.5
CT	B	2.7
SB2	B	2.6
SB11	B	1.3
V1	B	2.7
V2	B	2.7
ACA21	W	1.9
ACA22	W	1.9
ALKO1245	D	1.6
ALKO1523	D	1.3
ALKO743	B	3
ALKO554	B	1.3
ALKO1611	B	3
TS146	B	1.6
ATCC9080	Br	1.6
NCYC396	Br	2.2
B		
S. cerevisiae (*beticus*)	V	1.3–2
S. cerevisiae (*cheresiensis*)	V	1.3–2
S. cerevisiae (*montuliensis*)	V	1.7–2
S. cerevisiae (*rouxii*)	V	2

G, genetic line; B, bakers'; W, wine; Br, brewers'; D, distillers'; V, velum.
A: Modified from Codón et al. (1998); B: Modified from Martínez et al. (1995).

strains; on the other hand, different copies of a specific chromosome were not identical in wine strains (Carro and Piña 2001). These analyses also indicated that aneuploidies are widespread among wine strains, and suggested that *S. cerevisiae* wine yeasts are exceptionally tolerant to spontaneous loss or gain of chromosomes, but did not provide an explanation either for such widespread or for mechanisms accounting for it (Table 2). Genetic analysis of yeast hybrids derived from flor yeasts isolated from sherry wine and laboratory strains indicated that flor yeasts were also aneuploids, and that recombination occurred not only among homologous chromosomes of similar length but also among polymorphic partners with different sizes. Furthermore, polymorphism in chromosome length seemed to be a major source of karyotypic variation, as judged by the new chromosomal variants which were frequently observed in the meiotic products, recombinational events occurring both mitotically and meiotically (Guijo et al. 1997).

Mortimer et al. (1994) suggested that natural homothallic heterozygous wine yeast strains give rise to homozygous descendants, some of which may replace the original heterozygous parental. The process was called "genome renewal." However, sexual isolation in yeast population during wine production, the high level of heterozygosity and the low sporulation rates of wine yeasts (Table 3) do not favor this hypothesis. More recently, Puig et al. (2000) found mitotic rearrangements for chromosomes VIII and XII after 30 mitotic divisions, sporulation and meiosis were not involved in these processes. They suggested a process of gradual adaptation to vinification conditions, since chromosomal rearrangements and aneuploidies acquired following numerous mitotic divisions are maintained vegetatively, and mitotic recombination, instead of sporulation, would eliminate deleterious mutations and introduce genetic diversity. This suggestion does not mean that wine strains never sporulate, but that sporulation is not significant with regards to their genome evolution. The presence of lethal recessive mutations present in heterozygosis in wine yeasts support this view since the frequency of sporulation is lower than that of mutation (Jiménez and Benítez 1987). In spite of this isolation, breeding of strains with appropriate features has been carried out by protoplast fusion, single chromosome transfer, rare-mating, and transformation. Hybrid formation is isolated on selective media after crossing respiratory-deficient

Table 3 Genetic analysis of enological yeasts

Strains	Percentage of sporulation (20 days)	Spore viability (%)			Thallism[c]
		1st Gener[a]	2nd Gener[b]	3rd Gener[b]	
E-1	15.9	47.7 (11)	68.7	89.6	HOM
F-12	30.6	10.0 (20)	70.8	85.4	HOM
F-21	10.4	9.0 (45)	83.9	91.6	HOM/HET
F-23	16.6	23.2 (28)	31.2	41.6	HOM/HET
F-25	6.2	2.3 (22)	4.2	4.2	HOM?
G-1	8.3	0.0 (12)	<1.0	ND	HOM?

[a] The number of asci dissected is shown in parenthesis.
[b] Obtained either by selection of clones with good growth and sporulation, and posterior dissection of asci (E-1, F-12, and F-21 strains), or by obtaining random spores through treatment with diethyl ether (F-23, F-25, and G-1 strains).
[c] HOM, homothallic; HET, heterothallic; HOM/HET, heterozygous for the thallism. Modified from Guijo et al. (1997).

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mutants and auxotrophic strains (Codón and Benítez 1995). Single chromosome transfer can be achieved using *kar* mutants incapable of nuclear fusion after conjugation and after the cytoplasms have been mixed. At low frequency the zygotes yield progeny that have the nuclear genotype of one parent plus an extra chromosome of the other, as a result of defective nuclear fusions (Conde and Fink 1976).

3.2.2 Improvement of Yeast Abilities for Substrate Utilization

Most of the fermentation alcohol produced worldwide uses *S. cerevisiae*. The lactose-fermenting yeast *K. marxianus*, the amylolytic yeast *Schwanniomyces castelli* and some pentose-fermenting yeasts such as *P. stipitis* also play minor roles in ethanol production at present. Genetic engineering has been beneficial, as it has enabled *S. cerevisiae* to ferment recalcitrant materials, after transformation with a cellulase gene from *Trichoderma reesei*, glucoamylase genes from *Aspergillus awamori* or *S. cerevisiae* var. *diastaticus*, xylose isomerase genes from *Escherichia coli*, or *P. stipitis* or β-galactosidase and lactose permease genes from *K. lactis* (Walker 1998). Other approaches aimed at improving ethanol productivity by enhancing the glycolytic flux (overexpressing individual glycolytic enzyme genes). However, these attempts have met with little success in increasing the rate of ethanol production.

The efficiency of strains other than *Saccharomyces* with appropriate hydrolases like *Candida*, *Hansenula*, etc., for ethanol production is at standard levels at the first phase of fermentation. However, in *S. castelli* amylases and *C. wickerhamii* cellobiases are reversibly and noncompetitively inhibited by ethanol at fairly low ethanol concentrations (Spencer-Martin and van Uden 1987). Transfer of a certain hydrolase from an ethanol-sensitive strain to a tolerant one might give rise to a lack of activity of the heterologous product at ethanol concentrations inhibitory for the sensitive donor. When regulation of endo-xylanase production by *S. cerevisiae* strains transformed with the *XYN2* gene from *T. reesei* was investigated, the rate of xylanase production was severely reduced at 0.4 M ethanol (du Preez et al. 2001). However, when hybrids between *Candida* and *Saccharomyces* were formed by protoplast fusion, there was an increase in the ethanol-tolerance of the cellobiase activity, indicating an indirect cellobiase inhibition in the ethanol-sensitive parental (i.e., other enzymes involved in cellobiase synthesis) (Martín-Rendón et al. 1989).

3.2.3 Efficiency of Substrate Conversion

Saccharomyces cerevisiae may utilize a variety of carbon sources, but glucose and fructose are preferred. When one of these sugars is present, carbon catabolite repression occurs, and the enzymes required for utilization of the alternative carbon sources are either synthesized at low rates or not at all (Gancedo 1998). This effect, termed carbon catabolite repression may occur at the transcription level where most of the genes are regulated by the Mig1p and Snf1p proteins (Gancedo 1998; Lombardo et al. 1992). Mig1p is a transcriptional repressor of glucose-repressible genes involved in metabolic processes different from glucose fermentation, such as utilization of the alternative carbon sources sucrose, maltose or galactose, gluconeogenesis and respiratory metabolism; Snf1p is a cytosolic serine/threonine protein kinase that functions as a global regulator of derepression of glucose-repressible genes through Mig1p phosphorylation (Gancedo 1998); transcription of genes necessary to grow in nonfermentable carbon sources is activated by the Hap complex, which is repressed by Mig1p. Productivity may be increased by redirection of the respiro-fermentative flux through the deregulation of Mig1p or Hap complex (Blom et al. 2000).

In molasse fermentation whose main carbon source are sucrose and raffinose, modification of invertase expression would improve utilization of molasses. The expression of the *SUC* genes, which code for the invertase required for catabolism of sucrose and raffinose is repressed at high levels of glucose (Gancedo 1998). Different regulatory regions such as *SUC2A*, *SUC2B* (activation sequences), and upstream repression sequence (URS_{SUC2}) have been identified in the promoter of the *SUC2* gene. Whereas Mig1p binds to *SUC2*A and *SUC2* B in the presence of glucose, repression mediated by URS_{SUC2} occurs in the absence of glucose. *SUC2* is not repressed in *mig1* mutants (Treitel and Carlson 1995) while mutations in other genes act synergically with *mig1* or *snf1* on *SUC2* deregulation (Gancedo 1998). A deletion of an URS_{SUC2} region increases the maximum expression of *SUC2* from two- to eight-fold, depending on the yeast strain (Gancedo 1998). An increase in the copy number of the *SUC4* promoter also produces an increase in the expression of the invertase genes, suggesting that transcriptional regulatory (negative) factors may become limiting (Gozalbo 1992). Such a dilution effect could explain the high invertase levels found in industrial yeasts derived from sources containing sucrose, where the presence of many *SUC* genes located on different chromosomes were observed (Codón et al. 1998; Naumov et al. 1996; Ness and Aigle 1995). With regards to amylaceous substrates, maltose utilization requires a *MAL* locus and transcription of the structural genes for permease (*MALT*) and maltase (*MALS*) which are induced by maltose and catabolite-repressed by glucose (Higgins et al. 1999; Needlemann 1991; Yao et al. 1994). Both genes share a common intergenic upstream activation sequence (UAS) region whose activation results in the coordinate expression of *MALT* and *MALS* (Yao et al. 1994). Thus, glucose plays two roles in maltose utilization: it interferes with induction of the *MAL* transcriptional activator by maltose and represses the expression of the permease and the maltase (Gancedo 1998; Higgins et al. 1999). Glucose can also affect enzyme levels by decreasing the transcription rate, and by increasing the degradation rate of the corresponding proteins (catabolite inactivation) (Gancedo 1998).

2-deoxy-D-glucose (DOG), a toxic glucose analog has been frequently used to isolate glucose-deregulated mutants.

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Mutants isolated in galactose and DOG from industrial *S. cerevisiae* strains ferment equimolar mixtures of glucose and galactose to ethanol rapidly and completely (Bailey et al. 1982). In addition, deoxyglucose-resistant (DOG^R) mutants isolated on maltose or raffinose and DOG, with *MAL* and SUC deregulated phenotypes have improved leavening ability in doughs. This increased fermentative capacity correlated with increased maltase and invertase activity (Rincón et al. 2001).

3.2.4 Improvement of Ethanol Production

Important factor in yeast selection for ethanol production include: (a) absence of metabolites other than ethanol, (b) high osmotolerance and ethanol tolerance, and (c) high resistance to several physicochemical stresses (Spencer and Spencer 1997).

Saccharomyces cerevisiae yeasts producing higher amounts of ethanol at the expense of other by products such as glycerol, quantitatively the most important fermentation product after ethanol and carbon dioxide (Remize et al. 1999), would be of considerable value during fuel production. The possibilities of the diversion of carbon flux from glycerol towards ethanol have been investigated, so that yeast strains were engineered to produce larger amounts of ethanol with a lower glycerol yield. Disruption of GPD1 gene, which encodes a glycerol 3-phosphate dehydrogenase, resulted in a 2.5 fold decrease in glycerol production (Remize et al. 1999). In a similar way, modulation of HAP4 expression, a positive transcriptional regulator of genes involved in respiratory metabolism, could positively affect the balance between fermentation and respiration. The ethanol yield on fermentable carbon sources can be increased at the expense of biomass production (van Maris 2001).

There is usually no correlation between resistance to osmotic stress and ethanol tolerance. Furthermore, some strains that predominate in wines with high alcohol content (16%) are less osmotolerant than other wine yeasts, which are not ethanol-tolerant (Aranda et al. 2002). Ethanol-tolerance in yeast species is controlled by a large number of genes, so that it is difficult to isolate ethanol-tolerant strains by conventional screening and selection techniques. Continuous culture allows the accumulation of several mutations or the selection of ethanol-tolerant hybrids between nonisogenic yeast strains, which may give rise to more ethanol-tolerant strains. In fact, mutants of *S. uvarum* whose fermentation rate in ethanol is higher than that of the parental have been obtained in continuous culture with increasing ethanol concentrations (Figure 7) (Brown and Oliver 1983).

Complementation between nonisogenic strains has frequently produced hybrids with improved features with regards to their parentals (Jiménez and Benítez 1987). Hybrids between ethanol-tolerant strains of *S. cerevisiae* var. *diastaticus* and poorly ethanol-tolerant brewers' yeast were more ethanol-tolerant, had a faster fermentation rate and attenuated better than their parentals (Day et al. 1975) and were capable of using dextrins and starch as carbon sources (Figueroa et al. 1984); protoplast fusion products of *S. uvarum*

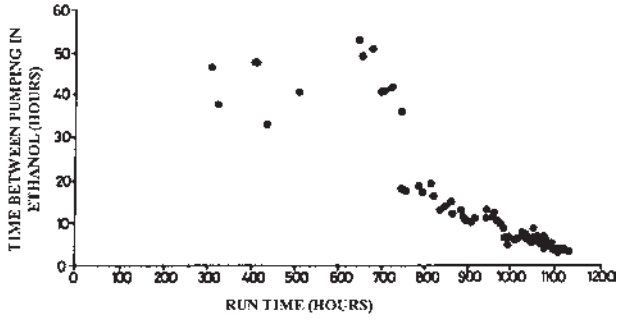

Figure 7 Improvement in the ethanol tolerance of the culture. The graph describes the frequency of switching of the ethanol pump in response to an increase in the CO_2 concentration of the exit gas. Each point represents a 7 h moving average of the interval between operation of the ethanol pump. (van Uden 1989).

and *S. cerevisiae* var. *diastaticus* had enhanced ethanol-producing capacity and increased thermotolerance and osmotolerance better than their parentals, and those of *S. cerevisiae* and *S. mellis* possessed increased osmotolerance.

Synergy between inhibitory effects of ethanol and other agents such as temperature desiccation and others have been studied, and strains, which simultaneously become resistant to ethanol and other hostile agents have been reported (Jiménez and Benítez 1988). Tolerance of yeast cells to ethanol or freezing lies within the cell membrane, so that the integrity of the membrane is critical to the success or failure of the yeast (Caron 2001). Membrane integrity during freeze, as occurs in the presence of high alcohol levels and under high osmotic pressure, requires a high phospholipid content to prevent it from breaking (Attfield 1997). The importance of trehalose not simply as a reserve carbohydrate but as a cell protectant during extreme stress situations such as desiccation and freezing has been reported (Caron 2001). Freeze-tolerant strains showed higher trehalose accumulating abilities than other strains, but once the cells started to ferment, this correlation was lost and maintenance of a high trehalose level did not suffice to prevent loss of stress resistance (Attfield 1997; Hino et al. 1990; Randez-Gil et al. 1999).

In some experiments conducted with ethanol-tolerant wine yeasts, improved fermentation performance was found in both lean and sweet doughs over a 6-month period (Caron 2001). Furthermore, transference to bakers' yeast of mitochondria, from strains highly tolerant to ethanol (Jiménez and Benítez 1988) gives rise to heteroplasmonts, which are more tolerant during storage at different temperatures and to freeze (Benítez et al. 1996): when viability of heteroplasmons was measured, it was slightly higher than that of the bakers' strains immediately after freezing the cells, and after one or few days. However, this improvement was lost and eventually heteroplasmonts were more sensitive than bakers' strains

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when the storage time at −20°C was prolonged. Drop in viability of heteroplasmonts was exclusively due to incompatibility between mitochondrial and nuclear genomes and not the result of recombination between mtDNA from different sources, because mtDNA restriction analysis of heteroplasmonts indicated the same mtDNA pattern as that of the donors (Codón et al. 1995). Wine strains may be genetically quite distinct from bakers' yeast, so that although they belong to the same species, *S. cerevisiae*, the lack of isogenicity accounts for the apparent incompatibility found between nuclei and mitochondria from different sources. The improvement found when wine mitochondria were transferred to laboratory strains (Jiménez and Benítez 1988) might indicate a greater proximity between wine and laboratory strains than between these and the bakers' strains. On the other hand, heteroplasmon formation using compatible yeasts may result in the isolation of stable strains more resistant to freezing and storage at different temperatures and more ethanol-tolerant.

Common heat shock and ethanol stress proteins with molecular masses varying from 23 to 70 kDa are induced in brewing strains in response to ethanol (Walker 1998) and up to 90 kDa in distillers' in response to either ethanol or acetaldehyde. Furthermore, heat and ethanol stress proteins lead to the acquisition of thermotolerance and ethanol tolerance (Walker 1998). Increase in MnSOD in response to ethanol has been interpreted as that ethanol induces oxygen free radicals and antioxidant enzymes, SOD play a defensive role in protecting cells from the damaging effects of ethanol. There is also a common pattern of expression of different HSP genes under several stress conditions, so that the levels of some of these genes induced by ethanol have been studied in flor yeasts highly ethanol-tolerant, able to survive in the surface of sherry wines with 16% ethanol. Resistance of flor yeasts to cold, osmotic, oxidative ethanol, and acetaldehyde stress correlated with induction of HSP12, HSP82, HSP26, and HSP104 genes. Furthermore, higher resistance to ethanol and acetaldehyde of some strains correlates with a higher induction of HSP genes and a predominance of these strains in the sherry (Aranda et al. 2002; Esteve-Zardoso et al. 2001).

4 CONCLUSIONS

(a) Ethanol acts as a noncompetitive inhibitor of yeast growth rate (μ) at relatively low concentrations. (b) Glycolytic metabolism is comparatively resistant to the inhibitory effects of ethanol. (c) Although intracellular ethanol increases as fermentation progresses because ethanol diffuses very rapidly across the cell membrane it does not accumulate in yeast cells. (d) Membrane structure and function are the predominant target of ethanol. The sites of ethanol action in yeasts are the plasma membrane, hydrophobic proteins of cell, mitochondrial membranes, nuclear membrane, vacuolar membrane, endoplasmic reticulum, and hydrophobic proteins in the cytoplasm. Exposure to ethanol results in increased membrane fluidity and consequential decreased membrane structural integrity. (e) Alcohol and heat both affect membrane lipids which play key roles in the stress physiology of yeast cells. (f) Inhibitory effects of ethanol are also enhanced synergistically by nutrient limitation and other metabolic by products such as acetaldehyde. (g) Adaptations in yeast which confer protection against ethanol include an increase in the proportion of sterols in the plasma membrane, the biosynthesis of heat shock-like proteins (ethanol stress proteins), elevated levels of trehalose and increased activity of MnSOD. (h) Ethanol-tolerance is under polygenic control. This and the complexity of ethanol inhibitory effects have hampered the isolation of more ethanol-tolerant strains. (i) Strains with improved ethanol-tolerance have been successfully isolated in continuous cultures with increasing ethanol concentrations.

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20

Solid-State Fermentation: An Overview

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1 INTRODUCTION

Solid-state fermentation (SSF) may be characterized as a fermentation process carried out on a solid medium with a low moisture content (A_w), typically 0.40–0.90 that occurs usually in a nonseptic and natural state (Nigam and Singh 1994). The SSF produces a high product concentration but has a relatively low energy requirement (Mudgett et al. 1992; Yang and Yuan 1990). Solid-state fermentation has been exploited for the production of fermented food (Bhumiratana et al. 1980; Heseltine 1983), and animal-feeds (GumbinaSaid 1996; Nigam and Singh 1996a; Sandhu and Joshi 1997). The production of ethanol as fuel (Hinman et al. 1992; Ingram et al. 1999; Lapadatescu and Bonnarme 1999), and the production of various enzymes including cellulolytic and lignolytic have been studied in SSF (Gombert et al. 1999; Nigam and Singh 1996b). Recently, SSF process has been applied for the bioremediation of textile effluent and dye degradation (Nigam et al. 2000; Robinson et al. 2001). Many of the solids used for SSF are unrefined and of agricultural origin (Balakrishnan and Pandey 1996; Durand et al. 1997) which makes the complete characterization and exact reproducibility of results difficult (Mitchell and Lonsane 1992). The SSF can be carried out on a variety of agricultural residues such as wheat straw, rice hulls, and corncobs. Solid-state fermentation has received greater interest from researchers, as several studies have demonstrated superior product yields and simplified downstream processing, following SSF (Barrios-Gonzalez et al. 1988; Maldonado and de Saad 1998).

Though in industry, secondary metabolites are mostly produced under submerged (SmF) conditions, mainly because the processes associated with scale-up are much simplified, compared to those required for scale-up in SSF (Table 1). Liquid fermentation also allows greater control of parameters, such as pH, heat, nutrient conditions, etc., but it must also be taken into account that scientists in the West have less experience with the SSF process, than their counterparts in the East (Johns 1992; Nigam and Singh 1994).

The use of SSF for protein enrichment of lignocellulose residues has received close attention due to its low level technology, reduced reactor volume per unit weight of substrate converted, and its direct applicability of the fermented product for feeding purposes. Large quantities of fibrous crop residues are currently underutilized as potential animal feed sources, especially in developing countries (Nigam and Singh 1996a). A major reason for this is the low protein content of the waste residues that can be enriched utilizing added urea as nitrogen source and subsequent fermentation by white-rot fungi, as demonstrated by Zadrazil and Brunnert (1980).

The use of SSF technology for the production of metabolites, including secondary metabolites, should not be discounted. The mycelial morphology associated with the microorganisms predominately used for secondary metabolite production is well suited for growth on a solid support and mycelial growth can have a detrimental effect on product formation if cultivated in viscous liquid media. The filamentous morphology of these fungal cultures and the secretion of metabolites into the submerged liquid growth media can further increase the viscosity of the fermenting liquid broth. Therefore, SSF technology can be exploited as an alternative to submerged culture by allowing better oxygen circulation (Elibol and Mavituna 1997).

This chapter describes the substrates and bioreactors used in SSF and the applicability of SSF for biotransformation and

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Table 1 Selection of secondary metabolites produced by SSF

Secondary metabolites	Microorganisms	Substrates	References
Aflatoxin	*A. niger*	Cassava	Barios-Gonzalez et al. (1990)
Antibiotic	*A. brassicola*	Corn and wheat	Hesseltine (1972)
Cephalosporin	*S. clavuligerus*, *C. aerominium*	Barley	Jermini and Demain (1989)
Ergot alkaloids	*C. fusitormis*, *C. pupae*	Bagasse	Hernandez et al. (1993)
Gibberellic acid	*F. monoliforme*, *G. fujikuroi*	Wheat bran	Kumar and Lonsane (1987a,b,c); Prema et al. (1988)
Iturin	*B. subtilus*	Okara	Ohno et al. (1992a,b; 1993; 1995; 1996)
Mycotoxin	*P. viridicatum*, *A. ochraceus*	Wheat, corn, and rice	Hesseltine (1972)
Penicillin	*P. chrysogenum*	Bagasse	Barrios-Gonzalez et al. (1988)
Surfactin	*B. subtilus*	Soya	Ohno et al. (1995)
Tetracycline, Clorotetracycline	*S. viridifaciens*	Sweet potato	Yang and Ling (1989)
Zearalenone	*F. monoliforme*	Corn	Hesseltine (1972)

production of various secondary metabolites, since recent advances indicate that bacteria and fungi, growing under SSF conditions are more than capable of supplying the growing demand for secondary metabolites.

2 AGRO-INDUSTRIAL/LIGNOCELLULOSIC SUBSTRATES FOR SSF

There has been an increasing trend towards more efficient utilization of agro-industrial residues such as cassava bagasse, sugarcane bagasse, sugarbeet pulp, coffee pulp and husk, wheat straw, corn-cob, and apple pomace, etc. Application of agro-industrial residues in bioprocesses provides alternative renewable substrates, and on the other hand it also helps in solving pollution problems due to their recycling and biotransformations. With the biotechnological innovations in the field of fermentation technology, such as renewed interest in SSF, many new applications have increased the potential of the better utilization of agro-industrial residues as the main fermentation-substrate. The composition of these substrates is lignocellulosic in nature. Lignocellulose is a compact, part crystalline structure that is compromised of the two most abundant polymers on earth, lignin and cellulose. Lignin units are arranged in a random, irregular, 3-D network that provides strength and structure and are very resistant to enzymatic degradation. On the other hand, cellulose is made up of a linear polymer chain that consists of a series of hydroglucose units in glucan chains. The hydroglucose units are held together by β1–4 glycosidic linkages producing a crystalline structure that can be broken down to monomeric sugars. Another major component of the lignocellulose structure is hemicellulose that is made up various polysaccharides. The function of hemicellulose has been proposed as a bonding agent between lignin and cellulose. Lignocellulosic wastes or residues are an ideal energy source if the two components can be successfully separated. Over 150 million tons of lignocellulose is produced annually as waste in United States and Canada alone, this material is generally considered waste and poses disposal problems. Lignocellulosic waste problems can be solved through utilization in SSF for the production of fuels, chemicals, and animal feeds.

3 SSF BIOREACTORS

Three basic groups of reactor are used for SSF, and these may be distinguished by type of mixing and aeration used. In laboratory scale, small-scale SSF occurs mainly in flasks and other reactors are used for larger scale product formation.

3.1 Tray Bioreactors

Tray bioreactors tend to be very simple in design with no forced aeration or mixing of the solid substrate. These

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reactors are restrictive in the amount of substrate that can be fermented, since only thin layers can be used to avoid overheating and maintenance of aerobic conditions (Tunga et al. 1999). Tray undersides are perforated to allow aeration of the solid substrate with each tray stacked vertically. In denoted reactors, temperature and relative humidity are the only controllable external parameters (Durand et al. 1997). Wooden trays were initially used for soy sauce production in Koji fermentations by *Aspergillus oryzae* (Pandey et al. 2001). The use of tray fermenters in large-scale production is limited, as they require a large operational area and tend to be labor intensive. The lack of adaptability of tray fermenters makes it an unattractive design for any large-scale production.

3.2 Drum Bioreactors

Drum bioreactors are designed to allow adequate aeration and mixing of the solid with limited damage to the inoculum or product. Mixing and/or aeration of the medium have been explored in two ways: by rotating the entire vessel or through the use of various agitation devices. Rotation or the use of agitation can be carried out on a continuous or periodic basis. In contrast to tray reactors, growth of the inoculum in drum bioreactors is considered to be better and more uniform. Increased sheer forces through mixing can, however, have a detrimental effect on the ultimate product yield. Although the mass heat transfer, aeration, and mixing of the substrate in drum fermenters is increased, damage to inoculum and heat build up through sheer forces may affect the final product yield. Application of drum reactors for large-scale fermentations also poses handling difficulties.

3.3 Packed Bed Bioreactors

Columns are usually constructed from glass or plastic with the solid substrate supported on a perforated base through which forced aeration is applied (Mitchell and Lonsane 1992), these reactors have been successfully used for the production of enzymes, organic acids, and secondary metabolites (Robinson et al. 2001). Forced aeration is generally applied at the bottom of the column with the humidity of the air kept high to avoid desiccation of the substrate. Disadvantages associated with packed bed column bioreactors for SSF include difficulties in retrieving the product, nonuniform growth, poor heat removal, and scale-up problems (Lonsane et al. 1985; Mitchell and Lonsane 1992).

4 ORGANIC ACID PRODUCTION

Organic acids are among the most common ingredients in the food and beverage industries, these compounds exhibit three properties; they are soluble, hygroscopic, and have buffering and chelation abilities. Organic acids that are mainly used as food acidulants, include; citric, lactic, acetic, malic, gluconic, propionic, and fumaric acids. In terms of volume produced, citric and acetic acids together, account for approximately three-quarters of food acidulant usage. Citric and lactic acid have been produced under SSF conditions for many years. Citric acid is manufactured at present entirely by fermentation even though there was great competition between microbial and chemical processes for various organic acid productions.

4.1 Citric Acid Production

The simplest way of producing citric acid is by SSF; various agro-industrial by-products being available for fermentation such as, coffee husk, wheat bran, cassava roots, etc. Substrates are generally moistened to around 70% of their adsorbance capacity and after a sterilization step, in order to reduce the potential of unwanted bacterial infection, inoculated with fungal spores. The time typically required for a citric acid under SSF conditions is circa 90 h. An important advantage of SSF over submerged fermentation for citric acid production is the presence of trace elements that does not affect the yield, as it does in submerged fermentations. Therefore, substrate pretreatment is not required; this saves both time and energy. In SSF, citric acid production is directly influenced by the nitrogen source. This nutrient is made available through the addition of urea, ammonium chloride, or ammonium sulfate. Utilization of nitrogen by the fungal inoculum leads to a decrease in pH that is favorable for citric acid production.

4.2 Lactic Acid Production

Lactic acid is produced by both fermentation (50%) and chemical synthesis (50%). It is widely used in the food industry as a taste-enhancing additive. In the pharmaceutical industry various L (+)-lactic acid salts are used for their therapeutic qualities. Production of L (+)-lactic acid by *Rhizopus oryzae* can be carried out under SSF conditions by using sugarcane bagasse as a substrate. On addition of a nutrient solution containing glucose and calcium carbonate, productivity rates up to 1.4 g/l/h can be achieved with yields in the range of 77% (Pandey et al. 2001).

5 COMPOSTING/PROTEIN-ENRICHMENT

The technology of SSF can be applied in a more effective way in the biotransformation and biological upgradation of crop and agricultural residues for improved and upgraded nutritional qualities (Zadrazil and Brunnert 1980). Composting is associated with edible mushroom cultivation and preparation of the growth media for this fermentation is extremely important. Properly prepared media for cultivation has a great impact on fungal mycelia colonization. Composting is carried out through the piling of solid for a period of time; during this phase various changes occur in

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the condition of the substrate. The natural compost is prepared from materials such as horse, cattle, or chicken manures. Synthetic manures are prepared from various agro-industrial waste products and residues, e.g., wheat and barley straw, rice-bran, saw dust, sugarcane bagasse, etc. (Mitchell and Lonsane 1992).

As in any SSF process, the moisture content of the solid substrate is crucial to the final product yield. A range of 55–70% of moisture is generally required for the optimal growth of most mushrooms. The compost must also have approximately 2% nitrogen content, with a C/N ratio of 17:1. Composting and other similar procedures have been successfully used for protein enrichment of lignocellulosic wastes; a process known as the "Karnal Process" has been carried out for the denoted purpose by Singh and Gupta (1996). The Karnal process is carried out in two stages and uses thin layered substrate (as in a tray fermenter) but without the use of a perforated tray support. In the first stage, the cereal straw is treated with 4% urea at a 40% moisture level and is ensiled under a polythene cover for 30 days. The second stage involves the use of a rectangular brick structure (200 × 150 cm) which acts as the bioreactor. The loosely stacked bricks help provide aeration from all sides with a thin layer of urea treated straw spread thinly inside the brick enclosure as a nitrogen source for the fungi. Subsequently, a *Coprinus fimetarius* innoculum is added to the compost and fungal growth enriches the protein content of the straw.

6 SECONDARY METABOLITE PRODUCTION

In industry, secondary metabolites are mostly produced under submerged conditions, mainly because the processes associated with scale-up are simplified compared to those required for scale-up of SSF. Liquid fermentation also allows greater control of operative parameters, such as pH, heat, nutrient conditions, etc. But the use of SSF technology for the production of secondary metabolites should not be discounted. The mycelia of microorganisms predominately used for secondary metabolite production are well suited for growth on a solid support due to their filamentous morphology. Therefore, SSF technology can be exploited as an alternative to SmF; the SSF provides better oxygen circulation (Elibol and Mavituna 1997) compared to viscous liquid fermentation media. Due to the lack of free water, smaller fermenters are required for SSF and, therefore, less effort is required in downstream processing. Wild-type strains of bacteria and fungi tend to perform better in SSF conditions compared to genetically modified microorganisms; these factors reduce the cost requirements for metabolite production (Barrios-Gonzalez et al. 1993).

6.1 Gibberellic Acid

Gibberellic acid (GA_3) is produced as a fungal secondary metabolite in the stationary phase of the growth cycle. GA_3 is widely used in agriculture and it is also of great economic and industrial importance. GA_3 may be used to end dormancy in seeds, promote flowering, or accelerate germination in the brewery industry (Balakrishnan and Pandey 1996). GA_3 was first produced by liquid fermentation using *Gibberella fujikuroi* or *Fusarium moniforme*. Production of GA_3 using SSF has recently been investigated for a number of reasons, particularly the high cost of GA_3 in SmF production with low yields and extensive downstream processing. The SSF technique has been shown to yield higher concentrations of GA_3 with minimal production and extraction costs (Balakrishnan and Pandey 1996; Bandelier et al. 1997; Tomasini et al. 1997). Bandelier et al. (1997) produced 300 mg GA_3/kg dry matter with a wheat bran substrate in a 10-day fermentation and yield of 240 mg/kg dry matter was obtained in a 36-h fermentation by Tomasini et al. (1997) on cassava. Both SSF fermentations exceeded the yield of 23 mg GA_3/l produced in a liquid fermentation studied by Tomasini et al. (1997) in 120 h. Balakrishnan and Pandey (1996) also compared yields in SSF and SmF; the results showed that SSF produced 3.5 times as much GA_3. Since, GA_3 has a widespread use, more efficient and higher product yields are desired. Small-scale SSF experiments show promising results with higher quantities being produced in shorter fermentation periods.

6.2 Ergot Alkaloids

Ergot alkaloids are produced by *Claviceps* spp. and some strains of *Aspergillus*, *Penicillium*, and *Rhizopus* spp. (Peraica et al. 1999). Alkaloids have been successfully used to treat diseases such as angina pectoris, hypertonia, enterics, migraine headaches, etc. (Demain 1999). Ergot alkaloids have also been used to treat infectious diseases like glaucoma and herpes zoster. Ergotamine tartarate is used as a pharmaceutical drug. Production of ergot alkaloids by SSF was demonstrated to be 3.9 times greater than that produced by *C. fusiformis* in SmF (Hernandez et al. 1993). The use of antifoam chemicals and the shear stresses caused by stirring prevented high yields in SmF. Better air circulation can also be achieved in SSF with increased yields (Balakrishnan and Pandey 1996). By removing the cost and trouble associated with antifoaming chemicals, and by maximizing yield production, SSF may be seen as a viable option for industrial scale production of ergot alkaloids.

6.3 Surfactin

Surfactin was first discovered in 1968 and it is one of the most powerful biosurfactants known. It is also used as a fibrin-clotting inhibitor (Arima et al. 1968) and can lyse erythrocytes and the spheroplasts and protoplasts of some bacteria (Ohno et al. 1995). Surfactin's surface activity (Cooper et al. 1981) and antibiotic activity (Bernheimer and Avigad 1970) increased demand for elevated production.

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The compound was conventionally produced under SmF conditions. However, the medium required by the *Bacillus* spp., although simple was expensive and this led to experimentation with cultivation under SSF conditions on cheap, renewable, agricultural by products (Peypoux et al. 1999). Surfactin is capable of reducing the surface tension of water from 72 to 27 nM/m^3 (Ohno et al. 1995), but it constitutes only a small fraction of the secondary metabolites produced by *Bacillus* spp. *B. subtilis* MTCC 2423 produces almost 1 g of biosurfactant/l in 96 h. The denoted observation has led to research into maximization of surfactin production by SSF. Demand for surfactin and higher yields by SSF suggest that large-scale production can be accomplished through this technology. A large global market could make the proposed SSF fermentation an economically attractive process.

6.4 Cephamycin C

Cephamycin C (Ceph C) is a broad spectrum β-lactam antibiotic produced by a variety of organisms, including *Nocardia lactamdurans*, *Streptomyces catteya*, and *S. clauverigerus*. It is used as a basic bulk drug for semisynthetics like cefotoxin and cefametadazole. Kota and Sridhar (1998) showed that Ceph C produced by SSF yielded a more stable antibiotic than the compound produced by SmF. Fermentation occurred on wheat rawa with production of Ceph C beginning on day 3 and reaching a maximum value after day 5. The addition of cottonseed de-oiled cake and corn steep increased yields further. Balakrishnan and Pandey (1996) also added starch supplements to achieve elevated yields. Temperature and pH conditions for the production of Ceph C were found to be the same as in SmF (Kota and Sridhar 1998), but maintaining a uniform temperature was shown to be difficult.

6.5 Penicillin

Penicillin was first produced by an isolate of *Penicillium notatum*, but today it is produced by a higher yielding isolate of *P. chrysogenum* (Balakrishnan and Pandey 1996; Penalva et al. 1998). The penicillins, including the cephalosporins, aminocillins, carbapencins, and mono-bactams, are all known as β-lactam antibiotics. A high yield of penicillin by SSF technology can be obtained in a short time period. Barrios-Gonzalez et al. (1993) illustrated how high quantities of penicillin could be produced by SSF in a short time period; this is a beneficial attribute when trying to produce one of the world's most widely used antibiotics. Under SmF conditions, a maximum value of 9.8 mg/l was recorded, while under SSF conditions 13 mg of penicillin/l was produced; these observations illustrate the economic advantage of SSF technology if they are optimized for large-scale production.

6.6 Cyclosporin A

Cyclosporin A (Cyc A) is a cyclic undecapeptide and was originally used as an antifungal agent; the compound is produced by *Fusarium solani*, *Neocosmospora varinfecta*, and *Tolypocladium inflatum* (Sekar and Balaraman 1998; Sekar et al. 1997). Cyclosporin A was found to have antifungal, antiparasitic, anti-inflammatory, and immuno-suppressive activity. It has been used successfully in heart, liver, and kidney transplant patients with high sales (Demain 1999), therefore the potential exists for increasing efficiency of Cyc A production under SSF conditions. Cyclosporin A has been produced mainly by SmF but can also be produced synthetically; the latter process is complex in comparison with SSF. Ramana Murthy et al. (1999) produced Cyc A under SSF conditions with a high yielding mutant of *T. inflatum*. Twice as much Cyc A has been produced on wheat substrate in SSF than into SmF (Balakrishnan and Pandey 1996). Solid-state fermentation provides a possibility to increase production of Cyc A. The use of SSF has been investigated for enhanced production of Cyc A on wheat bran as a solid substrate (Sekar and Balaraman 1998). The optimization of SSF parameters could lead to increasing Cyc A and other antibiotic yields still further.

6.7 Tetracyclines

Tetracyclines are broad-spectrum antibiotics that are mostly produced by SmF; these processes involve with a high energy input and elevated levels of waste. Tetracyclines can be used against gram positive and negative bacteria, coccidia, trichomonas, amoebae, mycoplasmas, and balantidii. Tetracyclines work by inhibiting the formation of the necessary complex of ribosome, mRNA, and aminoacyl tRNA. Yang and Ling (1989) showed that tetracycline was produced on day 3 of the fermentation on sweet potato residue. This value reached a maximum on days 5–6 and SSF was demonstrated to produce a stable antibiotic that had storage potential. Whereas tetracycline production in SmF decreases after prolonged fermentation due to cell autolysis.

6.8 Oxytetracyclines

Oxytetracycline produced under SSF conditions produced a more stable product. Yang and Wang (1996) showed that large mycelia and mycelial fragments in SSF had a positive correlation with the antibiotic yield; this observation provides a good indicator of antibiotic activity in SSF systems. Oxytetracycline production in SmF showed a decrease in yield due to cell autolysis.

6.9 Iturin

Iturin is an antifungal antibiotic produced by *Bacillus*, it has been shown in field tests to be effective against plant pathogens. Iturin is a cyclic heptapeptide of α-amino acids connected by a long chain β-amino acid (Ohno et al. 1992a,b; 1993; 1996). Ohno et al. (1992b; 1993; 1996) used soybean

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curd residue in SSF. This material is known as okara and traditionally incinerated as industrial waste. The potential of okara as a SSF substrate for the production of iturin was investigated by Ohno et al. (1993). The results showed that total iturin production was superior in SSF in comparison with SmF. It was also noted by Ohno et al. (1993) that iturin produced by SSF had an enhanced antibiotic activity; the elevated activity was attributed to the antibiotic having a longer side chain. Maximum iturin production in SSF occurred after day 2 (compared to day 5 in SmF) SSF was found to be 6–8 times more efficient in SSF than SmF (Balakrishnan and Pandey 1996; Ohno et al. 1992a). Ohno et al. (1992a) also illustrated that the iturin extraction was simplified in SSF and this process required less solvent. Makkar and Comeotra (1999) reported that the *B. subtilis* growth rate and iturin formation were greater when using industrial wastes rather than synthetic media in SSF.

7 CONCLUSIONS

Solid-state fermentation has a wide and varied use and spans across many fields of science from the ancient art of soy sauce production to the biodegradation of xenobiotic compounds. With a minimal water requirement other microorganisms are out competed and although the system is nonseptic, spoilage and contamination are limited. The use of SSF for the production of commercially valuable metabolites and also for protein enrichment is at present under utilized. The potential of SSF can be realized if scale-up parameters, such as cooling and heat transfer can be more easily controlled. The SmF is broadly used for the production of many metabolites because it is a familiar technique, scale-up from laboratory level to industrial fermenter is simplified and process parameters are more easily monitored and controlled. However, the use of SSF should be considered by industry, especially when large quantities of secondary metabolites are required in short fermentation periods with minimal expenditure on media and downstream processing. Although SSF may be a possible technology for increasing yields, problems arise when experiments are scaled-up. Although SSF produces a more highly concentrated product than SmF, it is diffused through the solid media and, therefore, extraction may be required. With the extraction of large quantities of solids more concentrated impurities are also extracted with the cost of purification being proportional to the concentration of inert compounds; this may actually lead to an increase in recovery costs, especially for the extraction of secondary metabolites, where purity of product is critical. With the absence of free water in SSF, a more concentrated product is recovered, but the difficulty of separating the solid media from the product may dismiss the advantage. As with any fermentation system, SSF has apparent flaws and drawbacks but also the process has great potential in the enrichment of waste residues for animal feeds, enzyme production, fuels, and also for bioremediation purposes. It is clear that SSF can be used for the successful production of secondary metabolites, such as antibiotics and also for the protein enrichment of lignocellulose wastes if reactor design and scale-up parameters can be refined and optimized.

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Basic Principles for the Production of Fungal Enzymes by Solid-State Fermentation

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1 INTRODUCTION

Enzyme production is a major field of contemporary biotechnology. Annual world sales of enzymes in 1998 were close to 1.3 billion US dollars with a predicted sales value of 2.2 billion for 2009, mainly as commodity enzymes, such as, "alkaline proteases (detergents), amylases/cellulases (textiles), amylases/glucoamylases/isomerases (starch) and rennet (dairy)" and also, for a variety of food processing purposes (see T. Godfrey, 2002, ET Consulting, U.K. http://www.biocatalysts.com/frames/Biocatalysts_01.html).

Filamentous fungi such as those belonging to the genera, *Aspergillus*, *Penicillium*, and *Fusarium* are becoming a major source of industrial enzymes, because they have the ability to produce and excrete large amounts of these catalysts. They are also easy to culture in conventional submerged (SmF) and solid-state fermentation (SSF) and can be used to over produce transgenic enzymes (Berka 1991). The use of SSF for enzyme production has recently been reviewed (Pandey et al. 1999; Shankaranand et al. 1992; Viniegra-González 1997) for a diverse range of enzymes and isolates, and the methods often give higher enzyme productivity as compared to conventional SmF techniques. The reason for such differences in enzyme productivity between SSF and SmF techniques are currently being studied (Pandey et al. 1999) but Viniegra-González et al. (2002) advanced the hypothesis that they are due to differences in the local micro-gradients of oxygen and substrate concentrations within the fungal aggregates formed on the surface of solid support or within the liquid of fungal suspensions. Whatever the reasons, there is a growing interest in the use of SSF technology for enzyme production because it uses less energy and water and has potential savings in other downstream processing expenditures, such as in enzyme purification. This chapter concentrates on the basic principles of enzyme production by SSF techniques, with emphasis on the interactions between microscopic and macroscopic features of the processes and the implication of these in relation to enzyme yield, as compared to conventional SmF techniques.

2 BRIEF HISTORICAL BACKGROUND

2.1 Koji Technology

One of the oldest techniques of producing enzymes by SSF is rice fermentation for the production of the rice wine called "sake" in Japan. Sake sales in Japan are estimated to be around $27 billion worldwide retail sales (http://www.sakeone.com). According to the "Sake Handbook" (http://www.sakeusa.com)" The first sign of sake brewing was found in the Yangtze Valley in what is now China, dated 4800 BC." A central part of sake brewing is the production by selected strains of *Aspergillus oryzae* or *Aspergillus awamori* of a crude amylolytic preparation (koji) which is a fermented mash of steamed rice. This process breaks down starch into glucose that is then fermented in a secondary stage by yeast (for more details see Aidoo et al. 1994; Hesseltine and Wang 1979; Hesseltine et al. 1988). Koji is also used to treat soybean paste to make miso soup. Production volumes of miso paste are estimated at around 300,000 tons per year (http://www.jadeesthetics.com), and koji fermentation is a full-fledged industrial technology in Japan. Traditional koji technology is, however, still practiced on a small scale at some miso factories of Japan.

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2.2 Early SSF Developments in United States

"Takamine (1894) applied for, and was granted, a patent titled 'Process of Making Diastatic Enzyme' (U.S. Patent 525,823)—the first patent on a microbial enzyme in the United States. He licensed his preparation to Parke, Davis & Company of Detroit under the brand name Taka-Diastase®. The company aggressively marketed the diastase as a digestive aid for the treatment of dyspepsia, which was said to be due to the incomplete digestion of starch" (see Bennet, The Timeline in (see http://pubs.acs.org/subscribe/journals). In 1899, the company Sankyo Shoten, a predecessor of Sankyo Co. Ltd. started to sell Taka-Diastase® made from the conventional koji fermentation with *Aspergillus oryzae* (http://www.sankyo.co.jp/general/I_info).

2.3 Blue Cheese Fermentation

One of the most well-known blue cheese fermentations is undertaken in the caves at the village of Roquefort, a few kilometers North of Montpellier, in France (see http://www.roquefort.fr). The traditional technology comes from the Middle Ages and Local tradition says that Charlemagne gave Roquefort the patent to produce their cheese. Nowadays, two companies sell several billion dollars worth of Roquefort cheese made with milk from some 750,000 ewes from neighboring regions of Spain, South France, and Northern Italy, that is transported to Roquefort in the form of salted curd. The curd is matured in Roquefort caves with selected strains of *Penicillium roquefortii*. Enzymes, such as proteases and lipases, produced by the fungas diffuse and help to transform the curd into a mature cheese with a distinct flavor and scent.

2.4 Moldy Bran

According to Prescott and Dunn (1959); Underkofler et al. (1947) developed an industrial technology called "moldy bran" for the production of enzymes using a SSF technique. This was based in a rotatory drum supplied with wheat bran

Table 1 Overview of the production of enzymes by SSF techniques[a]

Enzyme	Substrates
Cellulases	Cellulosic waste, sweet sorghum silage, coconut pith, sugar cane bagasse
Chitinase	Solid waste of the shellfish
Glucose oxidase	Wheat bran
Glucosidases and Amylases	Cassava meal, wheat bran, rice bran, banana waste. Starch, soya flour
Glutaminase and glutamate oxidase	Impregnated polystyrene beads, wheat bran, rice husk,
Inulinases	Wheat bran, rice bran, coconut oil cake and corn flour
Laccasses and peroxidases	Lignocellulosic fibers
Lipases	Wheat and rice bran, coconut cake, babassu oil waste.
Pectinases	Wheat bran, apple pomace, coffee pulp, sugar cane bagasse with pectin, soy bran, polyurethane with pectin.
Phytases	canola meal, wheat meal, mustard, cowpea and groundnut cakes, cowpea meal.
Proteases	Wheat and rice bran, rice chaff
Pullulanases	Wheat bran
Rennin	
Tannases	Sugar cane bagasse with tannic acid, forest residue, polyurethane with tannic acid.
Xylanases	Wheat bran; coffee waste; wheat or rice straw, beet pulp and apple pomace mixtures; coffee waste, ligno cellullosic fibers, sugar cane bagasse, banana, corn cob, mango peel.

[a] For more details see Pandey et al. (1999).

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fortified with a mineral solution. Moldy bran was used for the production of cellulases, amylases, and pectinases (see Table 1) and was considered an extension of koji technology. Upgrades of such technology will be discussed below.

2.5 Overview of Recent Work

Table 1 shows a partial compilation of processes for the production of enzymes by SSF methods. It is of note that nearly 20 enzymes, mostly hydrolases acting on carbohydrates, have been produced in a variety of solid supports. Most of the processes have used natural biodegradable supports such as wheat bran, sugar cane bagasse, beet pulp, and coffee wastes. However, some work has utilized artificial nonbiodegradable materials such as amberlite, polystyrene, and polyurethane. The major enzymes produced have been cellulases, glycosidases (including amylases), laccases and peroxidases, pectinases, proteases and xylanases. These findings suggest that a major subject of SSF studies is the breakdown of lignocellulosic fibers. This seems justified by the fact that such fibers are difficult to suspend and stir in high concentrations inside liquid fermentation systems and are natural substrates for many fungi. Overall, wheat bran seems to be the favorite solid support for enzyme production. This material has some attractive properties, namely, wide availability, low cost, high water retention, convenient texture (allowing air flow with low coalescence if stirred), and a good diversity of natural inducers, such as, starch, cellulose, hemicellulose, protein, and lipids. It is also worth noting that although SSF techniques have been used mostly with fungi, there are also a number of reports of enzyme production by SSF using bacteria and yeast (not shown in Table 1).

3 BRIEF SURVEY OF PRESENT INDUSTRIAL SSF SYSTEMS

3.1 Fixed Bed Koji Methods

Koji production is undertaken in shallow fixed beds of fermentation mash (less than 5 cm high), distributed in trays, instead of the traditional small bamboo boxes. Air supply and cooling is provided by natural convection currents between the mash and the environment, although the trays may be covered to prevent contamination. In this way, many trays can be put in stacks in large air-conditioned rooms. Despite the simplicity of this technology, it has some drawbacks. It requires a large component of manual labor, and is an open system where spores, or mycelial fragments are difficult to contain during the enzyme recovery step. Nevertheless, this type of tray-based approach has been adapted to a great variety of fermentation processes around the world and is used for enzyme production by AllTech Co. Ltd. based at Kentucky, United States. This company produces enzymes by an adaptation of the Japanese tray system at Ciudad Serdán, Puebla State, Mexico. They report production of nearly 150 tons per month of crude phytase preparations as feed additives and also produce koji type amylases (http://www.alltech-bio.com).

3.2 SSF Fermentation with Fixed Beds and Forced Aeration

One way to overcome the heat-exchange limitations of tray technology is the use of fixed beds with forced aeration (Raimbault 1980; Saucedo-Castañeda et al. 1990). This concept was extended by Roussos et al. (1993) using the Zymotis reactor, which is a series of vertical, parallel, heat exchanger plates with forced aeration through the fermentation mash. This allows for heat exchange to be at least partially dissociated, from the mass transfer of oxygen, water, and carbon dioxide. Mitchell and Von Meien (2000) have studied the heat transfer limitation of this kind of design, which has been applied for pilot-plant production of fungal biomass (Roussos et al. 1993) and spores (Roussos et al. 1991), and may be of some use for enzyme production.

Biocon India Ltd., based in Bangalore, India, adapted the Zymotis design to give a series of horizontal round trays, stacked on top of each other. This design was developed as a fully automated "proprietary bioreactor" (called the Plafractor TM" (http://www.biocon.com/html)) and was designed to be a self-contained system for the use of conventional or genetically modified organisms (Suryanarayan and Mazumdar 2001).

3.3 Solid Fermentation with Mechanical Stirring and Aeration

Another alternative to overcome the limitations of heat exchange in SSF systems is the use of mechanical stirring together with forced aeration. One example of this is the system of horizontal beds with mechanical stirring as developed by Durand and Chereau (1988) at the laboratories of Institut National de la Récherche Agronomique (INRA) at Dijon, France. Their collaborative research with Saint Louis Sucre led to the formation of a company called Lyven for marketing enzymes for winemaking (pectinases), bread making (xylanases, proteases, lipases, glucoamylases), feed applications (β-glucanases and xylanases) and textile treatments (amylases and cellulases).

The basic principles of horizontal rotating drums developed by Underkofler et al. (1947) have recently been reconsidered (Marsh et al. 2000; Schutyser et al. 2001). Such a process may have two major limitations: attrition of fungal growth and coalescence of solid particles. These problems, however, are dependant on the kind of solid support and the species of fungus used in each particular fermentation process. This brief review of the main alternatives for SSF technologies used for enzyme production shows that there is

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no standard model for this kind of process. Instead, a variety of models are applied depending on raw materials, organisms used, products and the consequent up-stream and down-stream limitations. It is therefore necessary to review the basic principles of enzyme production by fungi in SSF in order to consider reactor design.

4 BASIC PRINCIPLES FOR FUNGAL GROWTH ON SOLID SURFACES

4.1 Biochemical and Physical Limitations of Fungal Growth

4.1.1 Effect of Sugar Concentration on the Specific Growth Rate

Experimental data show that growth of *Aspergillus niger* follows a logarithmic relation when cultured on agar plates (Larralde-Corona et al. 1997). For example, if spores of *A. niger* are evenly dispersed on an agar plates, and biomass production is measured as the average surface biomass density, X^{A} (mg/cm^2), the kinetics of growth can be described by Eq. (1)

$$\frac{\mathrm{d}X^{A}}{\mathrm{d}t} = \mu_{\mathrm{M}}\left[1 - \frac{X^{A}}{X_{\mathrm{M}}^{\mathrm{A}}}\right]X^{\mathrm{A}} \tag{1}$$

where, $X_{\mathrm{M}}^{\mathrm{A}}$, is the equilibrium value for $X^{\mathrm{A}} > 0$, for which $\mathrm{d}X^{\mathrm{A}}/\mathrm{d}t = 0$, and, μ_{M}, is the start-up value of the specific growth rate, $(1/X^{\mathrm{A}})\mathrm{d}X^{\mathrm{A}}/\mathrm{d}t$, measured when $0 < X^{\mathrm{A}} \ll X_{\mathrm{M}}^{\mathrm{A}}$.

Parameters, X_{M}, and, μ_{M}, can be estimated using conventional least squares from growth curves as indicated by Larralde-Corona et al. (1997). They found that the values of, X_{M}, and, μ_{M}, when plotted against the initial level of glucose, S_0, can be approximately fitted to a Monod equation with substrate inhibition for, μ_{M} (Eq. (2))

$$\mu_{\mathrm{M}} = \frac{\mu_{\mathrm{GM}} S_0}{K_{\mathrm{GM}} + S_0 + \frac{S_0^2}{K_{\mathrm{GI}}}} \tag{2}$$

or without substrate inhibition for $X_{\mathrm{M}}^{\mathrm{A}}$ (Eq. (3)) as shown in Figure 1.

$$X_{\mathrm{M}}^{\mathrm{A}} = \frac{X_{\mathrm{M}}^{\mathrm{A0}} S_0}{K_{XS} + S_0} \tag{3}$$

4.1.2 Steric Hindrance of Fungal Growth in Porous Supports

Laukevics et al. (1985) Villegas et al. (1993) advanced the idea that a major constraint of fungal growth within a porous material is the steric or geometrical hindrance produced within the interstitial space when the mycelium starts to branch out and occupy that space.

A heterogeneous bioreactor packed with a solid support, supplied with glucose and mineral salts, and inoculated with a fungus using conventional chemical engineering techniques. For example, the porosity, α, of a reactor packed with a solid material having intrinsic (microscopic) solid density, $\rho_{\mathrm{S}}^{\mathrm{m}}$, and apparent (macroscopic) solid density, $\rho_{\mathrm{S}}^{\mathrm{M}} \leq \rho_{\mathrm{S}}^{\mathrm{m}}$, can be

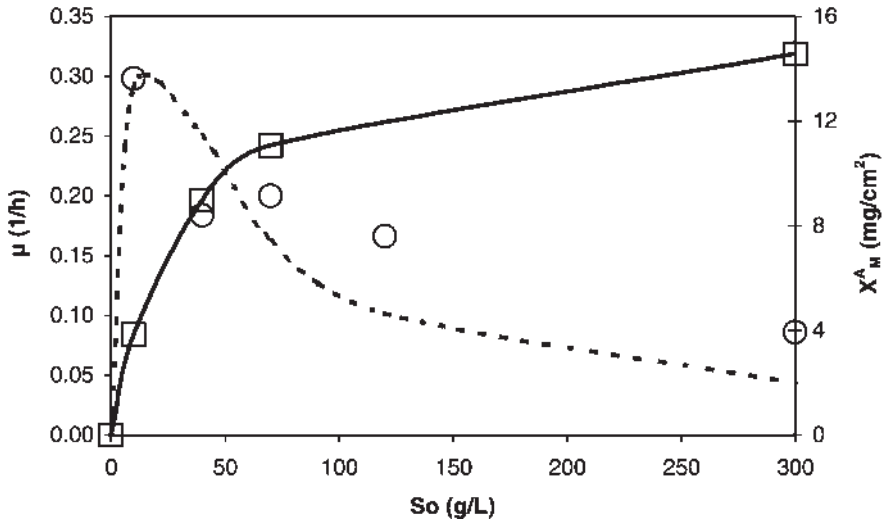

Figure 1 Effect of initial glucose concentration, S_0, on the maximal specific growth rate, μ_{M}, (○) and the maximal biomass surface density, X_{M}, (□) of *A. niger* N10, grown on agar plates (data from Larralde-Corona et al. 1993). Interrupted line is the best fit to experimental data, using Eq. (2) (see text) with $\mu_{\mathrm{M}}^0 = 0.92/\mathrm{h}$, $K_{\mathrm{S}} = 5\,\mathrm{g/L}$, and $K_{\mathrm{I}} = 15\,\mathrm{g/L}$. Continuous line is the best fit with Eq. (6) (see text) with $X_{\mathrm{M}}^0 = 16\,\mathrm{mg/cm^2}$, $K_{\mathrm{S}} = 32\,\mathrm{g/L}$.

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estimated by the following formula

$$\alpha = 1 - \frac{\rho_S^M}{\rho_S^m} \tag{4}$$

As indicated above, porosity, α, decreases when, $\rho_S^M \rightarrow \rho_S^m$. Figure 2 shows the decreasing trend of, ρ_S^M, as a function of the particle diameter, D, measured with different screens using cane bagasse particles packed at random. Hence, porosity (which is opposite to ρ_S^M) increases with particles with a larger diameter, D. For example, using the data in Figure 2, porosity (α) increased from 0.6 when $D = 0.1$ mm, to 0.8 when $D = 2.5$ mm (the intrinsic density ρ_S^m, of the bagasse powder was 1.2 g/cm^3 (Oriol 1987). This is related to the fact that the average microscopic surface area to volume ratio, r^m, of the support particles is an inverse function of diameter, D. The values of, r^m, can be obtained by measuring the geometry of representative particles using image analysis techniques. For example, for cylindrical rods of bagasse fibers generally have a length to diameter aspect, (β) of 5, when measured in from electronmicrographs (Saucedo-Castañeda 1991). Therefore, the surface area to volume ratio, A/V, can be estimated by the following formula.

$$r^m = \frac{A}{V} = \frac{(2\pi D^2/4 + \beta\pi D^2)}{\beta\pi D^3/4} = \frac{4(1/2+\beta)}{\beta D}$$
$$= 4.4D^{-1} \tag{5}$$

Solid biomass density, X^S, can be estimated as the ratio of the amount of biomass, Q_X, and the solid content of a given sample by the following formula

$$X^S \equiv \frac{Q_X}{S} \tag{6}$$

As $Q_X = X^A r_s^m (1-\alpha) V_T$, and $S = \rho_s^m (1-\alpha) V_T$, combining Eqs. (5) and (6) gives Eq. (7)

$$X^S = \frac{4X^A(1/2+\beta)}{\rho^m \beta D} \tag{7}$$

This demonstrates that X^S is a function of X^A, because the other parameters (β, r^m, ρ_S^m, D) are fixed properties of the solid support. The trend of, X^S ∞ shown in Figure 2, is parabolic as a function of diameter, D

$$X^S \approx a_0 + a_1 D - a_2 D^2 \tag{8}$$

For the data given in Figure 2 this gives maximum X^S of approximately 22 mg/g, for $D = 1$ mm.

Table 2, shows the values of X^A, X^R, and X^L, estimated or measured in different SSF systems with polyurethane and bagasse as solid supports, for various levels of sugar (S_0) and compared with the biomass concentration obtained in shake flasks, X^L. It is worth noticing that X^L values in SSF systems were double those obtained in SmF with loosely packed reactors ($\rho_S^M = 0.015$ g/cm^3) but the inverse was found when a tightly packed reactor ($\rho_S^M = 0.113$ g/cm^3) was used. The values of X^A, were higher for the loosely packed reactors.

There appear to be two opposing effects when D is varied. Increasing D leads to an increase in porosity (α) because the packing density is reduced (Eq. (4)). In such a case, more interstitial space is available for growth. In addition, increasing D decreases the surface area to volume ratio, (r^m), reducing the availability of surface area fungal growth. These factors could be the reason that the curve, X^S, is a parabolic function of the diameter as shown in Figure 2. It should be noted that one of the adverse effects of increasing reactor biomass concentration (X^V), is the reduction of the diffusivity of oxygen and carbon dioxide (Auria et al. 1992), this increases the constraints to mass transfer within the mycelial mat and may be the basic mechanism of so-called steric hindrances.

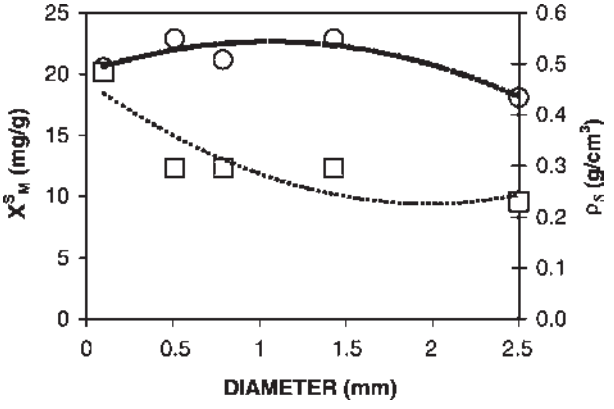

Figure 2 Effect of the diameter of washed out bagasse particles on the maximal solid biomass concentration, X_M^S (○, mg/g dry basis) and bagasse (macroscopic) packing density, ρ_S^M (□, g/cm^3) of a culture of *A. niger* N10, grown in small cylinders (2 cm × 20 cm) with forced aeration, supplied with a glucose solution, $S_0 = 280$ g/l fortified with minerals (C/N = 10; C/P = 120); $\omega = 2.8$ ml H_2O/g; inoculated with 2×10^7 jspores/g bagasse (data from Oriol 1987).

4.1.3 Effect of Sugar Concentration on the Maximum Biomass Concentration

Romero-Gómez (2001) studied the growth curves of *A. niger* cultured inside light polyurethane matrices, having a porosity (α) of 0.988, and an apparent macroscopic or apparent packing density, (ρ_S^M) of 15 mg/cm^3 (notice that a finely ground and tightly packed powder of polyurethane has a microscopic density of 1.2 g/cm^3). He found that such a polymer withstands a water load, (ω) of 20 g_{H20}/g_S in such a way that in a gram of polyurethane, water is evenly distributed in a shallow layer of depth (h) 62 ± 9 μm, with a surface area, (A) of 3225 cm^2, and a reactor volume (V_T) of 66.7 cm^3, gives a macroscopic surface area to volume ratio, (r^M) of 48.4/cm and a microscopic ratio, (r^m) of 153/cm. The

Table 2 Comparison of measured and estimated biomass densities, X_M, in various SmF and SSF systems (see glossary for notation)

	SSF					SmF	
ρ_s (g/cm^3)	r^M (1/cm)	S_o (g/L)	X_V^M (mg/cm^3)	X_M^S (mg/g)	X_A^M (mg/cm^2)	X_V^M (mg/cm^3)	Reference
0.018[a]	161[a]	55[a]	24.2[a]	484	3.61	10.9[a]	1
0.018[a]	161[a]	100[a]	35.7[a]	714	5.32	14.2[a]	2
0.113[a]	161[a]	50[a]	7.1[a]	142	1.05	14.4[a]	3
0.106[a]	44[a]	240[a]	11.9	33[a]	0.85	ND	4
0.018	161		150.0	3000	22.4	150	Theory[b]

References: (1) Diaz-Godínez et al. (2001); (2) Romero-Gómez (2001); (3) Aguilar (2001); (4) Oriol (1987).
[a] Measured quantities, others are estimates (see text).
[b] Estimated, assuming all the liquid is occupied by biomass with 15% solid content.

measured volumetric biomass density of *A. niger*, X_M^L, (estimated in terms of the liquid broth) with an initial glucose concentration of 200 g/l, was around 25 mg/cm^3 (Figure 2). This gave a surface biomass density. (X_M^A), of 0.16 mg/cm^2 (Table 2) which is nearly 16% of the estimated value for X_M^A of 0.96 mg/cm^2, assuming that all liquid is occupied by a tightly packed mycelium with 15% solid content ($X_M^V = 150\,\text{mg/cm}^3$) as shown in Table 2.

A plot of X_M^L (g/l) vs. S_0, for *A. niger* grown by SSF on polyurethane gives a straight line ($R^2 = 0.9979$), see Figure 3, and this is defined by Eq. (9)

$$X_M^L = 0.353 S_0 + 0.290 \tag{9}$$

whereas, the same type of plot for SmF (shake flasks) corresponded to a saturation curve (Eq. (2), but with X^L, instead of X^A variable) with parameters $X_{OM}^L = 23.5$ g/l; $K_S = 60.4$ g/l; $R^2 = 0.9936$, as indicated by Romero-Gómez (2001). This is strong evidence that SmF cultures seem to be limited by oxygen supply when the oxygen demand goes beyond 10 g/l, and SSF cultures, do not seem to be limited by oxygen supply even though oxygen demand is as high as $S_0 = 100$ g/l.

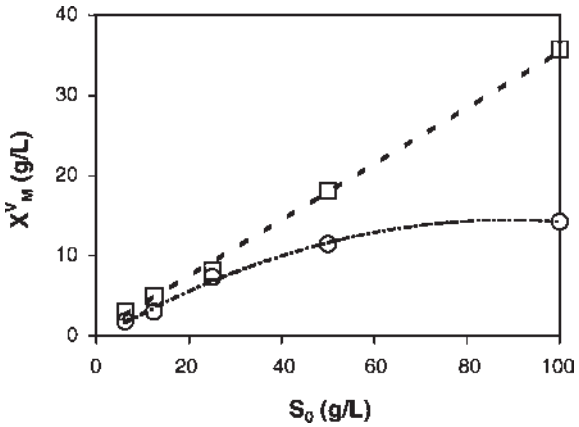

Figure 3 Effect of initial glucose concentration (S_0) on the maximal volumetric biomass density, X_M^V (see text for notation) of *A. niger* strain C28B25 in, submerged, SmF (○) or solid-state SSF (□) fermentation. Curves correspond to straight line ($y = 0.3531x + 0.2901 R^2 = 0.9979$) for SSF or Monod model ($X_{0M}^V = 23.5$ g/l; $K_S = 60.4$ g/l; $R^2 = 0.9936$) for SmF. Solid support was polyurethane. Experiments by SmF technique were done in shake flasks (data from Romero-Gómez 2001).

This feature is an inherent advantage of SSF systems over SmF systems because SSF systems do not seem to require stirring or mixing when they are undertaken in shallow layers of porous materials. The high levels of biomass achieved by Romero-Gómez et al. (2000) suggest that such a system is adequate for enzyme production that is associated to biomass production. In summary, the available results suggest that SSF systems produce good biomass yields when they meet the following specifications.

(a) High level of water load ($\omega > 10\,g_{H20}/g_S$) associated to high levels of water activity ($a_w \approx 0.99$); (b) High value of microscopic A/V ratio ($r^m > 10$/cm) of the solid support in order to disperse the liquid broth in shallow layers, preventing clogging of the interstitial space; (c) High porosity ($\alpha > 0.8$) to reduce steric hindrances for fungal growth and to enhance oxygen mass transfer in order to stand a very high level of biological oxygen demand ($S_0 > 0$) with a very high efficiency for biomass production (35%).

4.1.4 Microscopic Model of Fungal Growth

An important consideration for SSF systems is the geometry of fungal growth related to the average length (L_{av}) of the mycelial segments as this is a fundamental factor in relation to biomass packing density. Similarly the length of cylindrical fibers modifies the microscopic packing density (ρ_S^m) as shown in Figure 2. The relation between, L_{av}, and the specific growth rate, μ_M, is given by Eq. (10) derived by Larralde-Corona et al. (1997)

$$\mu_M = \frac{2\ln(2) U_M}{(L_{av} - d_0) + K_L \ln\left(\frac{L_{av}}{d_o}\right)} \tag{10}$$

where, U_M (μmh^1) is the maximal rate of apical extension, d_0

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(μmh^{-1}) is the average diameter of mycelial branches, and, K_L is an empirical parameter.

Eq. (10) has been used for the comparison, on agar plates, of pectinase over-producing mutants of *A. niger* (Loera-Corral and Viniegra-González 1998). They estimated, μ_M, for each given strain, using microscopic data processed by image analysis. They then used this data to classify mutants according to their resistance to low water activity and their relation to the production of pectinase, on agar plates. It is worth noticing that Eq. (10) depends on two seemingly independent factors, the rate of apical extension, and the average hyphal length, L_{av}. As a result two different fungal strains with equal U_R value, will have different μ_M values if their branching frequency described by Eq. (10), yields different values due to differences in L_{av}. For example, a highly branched strain of *A. niger* will have a low value of, L_{av}, and will form a dense mycelium on the surface area of a solid support. Conversely, a sparsely branched strain will have a high L_{av} value, with a loosely packed mycelium grown on the solid support. This shows the importance of considering both the physical structure of the solid support together with the branching pattern of the filamentous fungus to be used in an SSF technique.

4.2 Macroscopic Limitations of Fungal Growth on Solid Support

The practical importance of the previous considerations on fungal growth can be illustrated by an estimation of the bed size in a tray system. For example, if it is necessary to produce 100 kg of biomass (Q_X) from ground bagasse inoculated with *A. niger* as in the experiments illustrated by Figure 2. Then, it is possible to estimate the total volume required, V_T, as follows

$$V_T = \frac{Q_X}{(1-\alpha)\rho_s^m X^S} = 45.4\,m^3 \tag{11}$$

Considering an average bed thickness of 2.5 cm, the total tray surface area is 1,820 m^2. In contrast for a 0.5 m fixed bed with forced aeration (Zymotis type), the area would be only 91 m^2.

This estimate shows that one of the major macroscopic constraints for SSF technology is the macroscopic surface area of the fermentation mash. This is one reason why the traditional tray system using shallow beds ($h < 5$ cm) is difficult to scale-up due to the number of trays required to be stacked one on top of each other. Thus, a typical factory with the tray system will have to do fermentation batches with thousands of trays in order to achieve an output of several tons of fermented mash. Labor costs of the tray system can be decreased by automation using large round stainless steel plates that are stacked on in top of each other, and in such systems, a single fermentation tower will have hundreds of square meters of fermentation mash. This is a feature of the Plafractor® technology used by Biocon (India). In Europe, where labor costs are significant, one way to scale-up SSF technology is by mechanical aeration. One example of this is the use of deep fermentation systems with mechanical stirring in the Lyven factory in France.

Reactor volumes estimated for SmF and SSF systems are not comparable in terms of capital investment because in many cases SSF systems require simpler reactors without mechanical stirring and can be made of a great variety of materials. In many SmF systems stirred vats made of stainless steel are specified and these are expensive. Thus, in order to

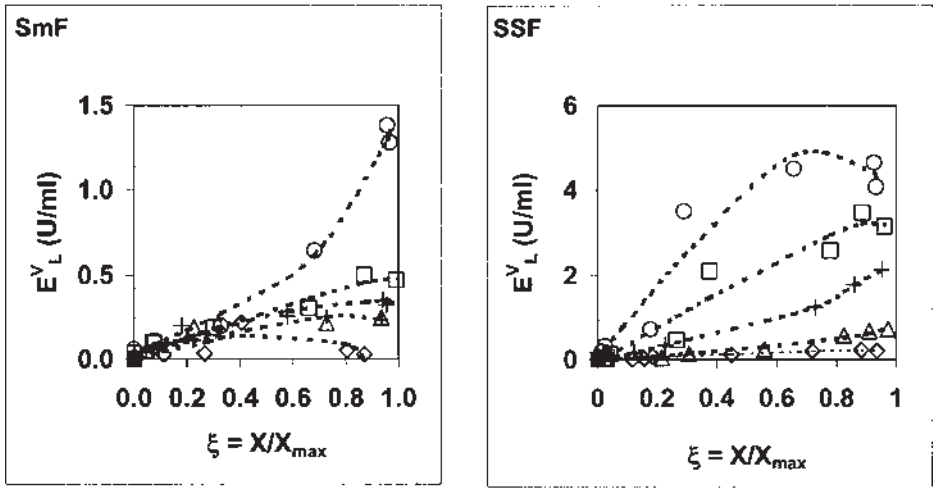

Figure 4 Effect of initial sucrose concentration (S_0) on the plot of E vs. $\xi = X/X_M$, of invertase, produced by *A. niger* C28B25 grown by SmF in shake flasks or by SSF on polyurethane foam. Initial values of S_0 (g/l) were, (◇) 6.25; (Δ) 12.5 (+) 25 (□) 50 (○) 100. Interrupted lines were the least square fit by equation 18. Data taken from Romero-Gómez (2001) Viniegra-Gonzalez et al. (2002).

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compare the SmF and SSF systems it is important to be specific about the exact design involved in each case. The scale-up principles of fixed or stirred beds with aeration have been widely reported and do not fall in the remit of this chapter. However, recent reviews include those by Durand and Chereau (1988) Oostra et al. (2000) Marsh et al. (2000) Pandey and Radhakrishnan (1992) Saucedo-Castañeda et al. (1990) and Roussos et al. (1993).

4.3 Modeling of Enzyme Production Between SSF and SmF Techniques

Enzyme production can be modeled from Eq. (12) proposed by Luedeking and Piret (1959)

$$\frac{dE}{dt} = Y_{E/X}\frac{dX}{dt} + kX \tag{12}$$

Where $Y_{E/X}$ (U/g) is the enzyme yield in terms of biomass production and k (U/gh) is the secondary rate coefficient of enzyme production, if $k > 0$. It is possible to combine Eq. (12) together with Eq. (1) (Viniegra-González et al. 2002) to give Eq. (13)

$$E(X) = E_0 + Y_{E/X}(X - X_0) + \left(\frac{kX_M}{\mu_M}\right)\ln\left[\frac{X_M - X_0}{X_M - X}\right] \tag{13}$$

where E_0, is the initial enzyme concentration and the biomass density (X) can be expressed for convenience in terms of surface area (X^A), liquid broth (X^V), or solid content (X^S). Now, defining the relative level of biomass $\xi = X/X_M$, Eq. (13) can be transformed into Eq. (14).

$$\Delta E = E - E_0 = Y_{E/X}X_M\left\{(\xi - \xi_0) + \gamma\ln\left[\frac{1-\xi_0}{1-\xi}\right]\right\} \tag{14}$$

where, $\gamma = k/Y_{E/X}\mu_M$, is the dimensionless ratio between secondary and primary rates of change of enzyme. Hence, the concentration of enzyme, $E(\xi,t)$, is proportional to the group, $Y_{E/X}X_M$. Figure 4 shows the comparison of invertase production plotted according to Eq. (14) in SmF and SSF cultures of *A. niger*. When, $\gamma = 0$, the enzyme is produced only in association with fungal growth (without apparent destruction). In such a case, the plot, ΔE vs. ξ, is a straight line with slope, $Y_{E/X}X_M$ (□ in Figure 4). When, $Y_{E/X} > 0$ and $k < 0$, the plot ΔE vs. ξ, has a maximum whenever, $-1 < \gamma < 0$, (□ in Figure 4 for SSF) and this maximum is obtained when, $0 < \xi = 1 + \gamma < 1$. This case corresponds to enzyme production associated to the growth process but accompanied by a fixed rate of breakdown, k. If some of the enzyme production occurs dissociated from the growth process, it implies that, $k > 0$, and is reflected as a concave curve (○ in Figure 4 for SmF). There is a remarkable difference in the enzyme titers shown in Figure 4 because, in SSF experiments, $E_S^V \leq 5.0\,U/cm^3$, whereas in SmF experiments, $E_L^V \leq 1.5\,U/cm^3$. Also, the shapes of the curves change in different manners for each type of culture. In SmF experiments, increasing concentrations of sucrose shifted the curves from convex ($k < 0$) to concave ($k > 0$) while the opposite was seen for SSF experiments (Figure 4). Therefore, the plot, ΔE vs. ξ, illustrated in Figure 4 is quite useful to classify the actual mechanism of enzyme production and also provides regression curves for the estimation of the parameters $Y_{E/X}$ and k (Viniegra-González et al. 2002), and this is illustrated in Table 3.

Table 3 shows the effect of the initial sucrose concentration (S_0) on parameters, $Y_{E/X}$, k, and γ, estimated by Eq. (14) from the data given in Figure 4. Despite the fact that standard deviations of $Y_{E/X}$ and k are rather high, Figure 5 shows that the trend of $Y_{E/X}$ vs. S_0 is completely different between SmF and SSF experiments. For SmF experiments, $Y_{E/X}$, has a definite decreasing trend on S_0, and for SSF, it is the other way around.

Studies of *A. niger* producing tannase (Aguilar et al. 2001) or pectinase (Díaz-Godínez et al. 2001) by SSF techniques, showed higher values of group $Y_{E/X}X_M$, and lower rates of enzyme destruction ($\nu \to 0$) as compared to lower values of

Table 3 The effect of initial sucrose concentration, S_0, on the parameters for enzyme production as defined by Luedeking and Piret and logistic equations (see text)

Submerged fermentation (SmF)				Solid state fermentation (SSF)			
S_0 (g/L)	$Y_{E/X}$ (U/g)	k (U/gh)	γ	S_0 (g/L)	$Y_{E/X}$ (U/g)	k (U/gh)	γ
6.25	307	−11.24	−0.39	6.25	113	−1.75	0.10
12.50	116	−2.08	−0.10	12.50	43	7.49	1.06
25.00	65	−0.96	−0.10	25.00	188	5.58	0.12
50.00	45	−0.13	−0.02	50.00	233	−1.45	0.03
100.00	63	1.18	0.14	100.00	335	−17.77	0.24
Means	119	−2.65	−0.10		182.33	−1.58	0.16
Std. Dev.	108	4.95	0.19		112.03	9.94	0.52

Data from Romero-Gómez (2001) illustrated in Figure 4.

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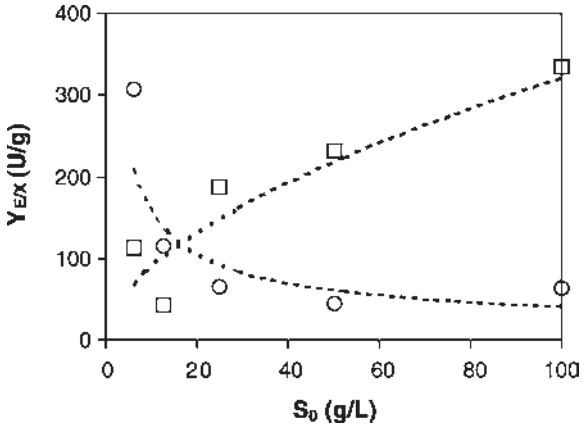

Figure 5 Effect of initial sucrose concentration (S_0) on the biomass yield of invertase, $Y_{E/X}$ produced by *A. niger* C28B25 grown by SmF in shake flasks (○) or by SSF on polyurethane foam (□). Interrupted lines were the least square fit by equation $y = Ax^{\beta}$. For SmF, $A = 615$, $\beta = -0.59$, $R^2 = 0.737$. For SSF, $A = 25$, $\beta = -0.56$, $R^2 = 0.589$. Data from Romero-Gómez (2001) illustrated in this figure and shown in Table 4.

group $Y_{E/X}X_M$, and higher rates of enzyme destruction ($v \rightarrow -1$) observed when same strains of *A. niger* are grown by SmF technique. The net result is that enzyme titers in SSF are much higher than in SmF experiments (see Table 4). This physiological observation has been confirmed by a number of laboratories (Table 4). Part of the better performance of SSF cultures has been attributed to some degree of resistance of *A. niger* to catabolite repression when cultured by SSF techniques.

4.4 Comparison of Enzyme Production by SmF and SSF Systems

Table 4 shows how enzyme titers of enzymes produced by SmF and SSF techniques can be compared to each other. For SSF systems, the enzyme titer in the leachate was calculated as E_L^V (U/ml) and the corresponding leachate volume, V_L, is shown. Enzyme solid concentrations, E, are also shown in Table 4, together with the enzyme titer per reactor volume (E^R). For SmF systems, the primary data were the liquid enzyme titers, E_L^V. Estimated reactor titers, E_R^V, were calculated assuming 25% headspace in the fermenters.

The ratios of liquid enzyme titers, $E_L^V(\text{SSF})/E_L^V(\text{SmF})$, estimated in Table 4 were higher only for those cases where the ratio $E^S(\text{SSF})/E^L(\text{SmF}) > 10\,\text{ml/g}$. This seems to suggest a simple rule of thumb for selecting SSF systems over SmF systems, if the enzyme is to be leached out for further purification and, is related to the fact that addition of leaching solvent will decrease the final enzyme titers produced by SSF system.

Comparison of ratios of enzyme titers per unit or reactor volume ($E^R(\text{SSF})/E^R(\text{SmF})$) shown in Table 4 seem to favor on the whole SSF systems, except in one case of

Table 4 Comparison of enzyme titers between SmF and SSF techniques

Enzyme	Extract volume A	E_L^V (U/mL) SmF (B to C) B	E_R^V (U/cm^3) SmF (B to C) C	E_L^V (U/mL) SSF (A and D to F) D	E^S (U/g) SSF (A and D to F) E	E_R^V (U/cm^3) SSF (A and D to F) F	$R(E_L^V)$ D/B	$R(E_R^V)$ F/C	Reference
Pectinase	5 mL/g dm	—	—	18	107	19.3	—	—	1
Phytase	20 mL/g dm	8.09	6.47	65.27	718	107.7	8.1	16.7	2
Cellulase	0.59 mL/g dm	—	—	54.3	233	18.2	—	—	3
Cellulase	0.59 mL/g dm	—	—	4.26	17.4	1.4	—	—	3
Endo-Pectinase	1 mL/g wm	0.8	0.64	2.54	14.5	1.3	3.2	2.04	4
Exo-Pectinase	1 mL/g wm	5.2	4.16	1.474	28	2.5	0.3	0.61	4
Pectin lyase	12 mL/g dm	0.178	0.143	0.468	5.6	8.4	2.6	59	5
Pectin esterase	12 mL/g dm	0.014	0.011	0.019	0.23	0.35	1.4	31.4	5
Poly galacturonase	12 mL/g dm	2	1.6	0.458	5.5	8.25	0.23	5.2	5
Leucine amino peptidase	—	4.3	—	—	17	—	—	—	6
Acid carboxi peptidase	—	0.09	—	—	16	—	—	—	6
Neutral protease	—	550	—	—	3,400	—	—	—	6
Acid protease	—	11	—	—	260	—	—	—	6
α-amylase	—	640	—	—	19,000	—	—	—	6
Pectinase	—	—	—	—	28	—	—	—	6
CMCase	—	1.4	—	—	87	—	—	—	6

References: (1) Castilho et al. (2000); (2) Papagianni et al. (1999); (3) Roussos et al. (1992); (4) Solís-Pereyra et al. (1993); (5) Taragano and Pilosof (1999); (6) Nam Soo et al. (2001).

exo-pectinase production by *A. niger* on sugar cane bagasse (Solís-Pereira et al. 1993), where $E^{S}(\mathrm{SSF})/E_{L}^{V}$ (SmF) was 5.4 ml/g. Altogether, it seems clear that SSF systems work best for enzyme production when two cases are met, (a) solid enzyme titers are high in the solid system. For example if $E^{S}(\mathrm{SSF})/E^{L}(\mathrm{SmF}) > 10\,\mathrm{ml/g}$, perhaps because the substrate is solid or the strain is highly oriented toward SSF process (Shankaranand et al. 1992) and (b) the final product is made of a solid residue containing the enzyme activities and the residual substrate. In the first case, it is necessary to have an important investment in strain improvement and process optimization in order to overcome the dilution problems associated with conventional liquid/solid extraction procedures.

In the second case, it is advisable to be sure that the final solid presentation has marketing or processing advantages over the pure enzyme. For example, by providing various enzymes that cooperate synergistically in a particular process, or when the residual substrate can be used as a raw material by the final user, either as a food, feed, or agricultural additive. In this case, the disposal of an agricultural residue used as a solid support will be an added environmental bonus to the process.

There is, however, room to improve processes that meet the first condition because of the unusual properties of high levels of aeration with low energy expenditures in SSF systems. This will be true if the very high titers of enzyme (E^{S} and E_{L}^{V}) are associated with specific physiological conditions of enzyme production, such as for example, high oxygen demands of the fermentation broth.

Comparing the mean values of E^{S} ($\approx 1000\,\mathrm{U/g}$) obtained by SSF as shown in Table 4 with the mean values of X_{M}^{S} ($\approx 0.3\,\mathrm{g/g}$) shown in Table 2, it is possible to assume that good strains for SSF systems will have a $Y_{E/X} \geq 3000\,\mathrm{U/g}$. In SmF systems, commercial processes for α-amylase production have $E^{V} \geq 100,000\,\mathrm{U/L}$. Table 4 includes a report from Nam Soo (2001) of a *Bacillus subtilis* strain grown on wheat bran and yielding $E^{S} = 19,000\,\mathrm{U/g}$. Thus, assuming $X_{M}^{S} \approx 0.3\,\mathrm{g/g}$, the estimated yield coefficient $Y_{E/X}$ would be $\approx 60{,}000\,\mathrm{U/g}$. This shows the importance of strain improvement, since wild strains of Fungi usually have values $Y_{E/X} \approx 300\,\mathrm{U/g}$ as shown in Table 3.

5 CONCLUSIONS

This chapter shows that the ancient art of SSF for the production of enzymes is being reconsidered by some companies around the world, using current research results on the physiology and engineering of this kind of process. Most of the recent research is focused on a variety of organisms (mainly mitosporic fungi), solid supports (mainly natural fibers such as wheat bran) and reactor configurations (trays and fixed beds with aeration or stirred fermented mashes). However the best way to proceed will also depend on particular local and economic conditions. The most challenging questions related to the physiology of SSF systems are related to the relation between the microscopic and macroscopic reaction systems because fungi operate as catalysts. However, much information has been accumulated by using artificial solid supports such as amberlite and polyurethane as this allows the measurement of biomass and enzyme production and the comparison of both variables to the physical and chemical nature of the substrates. Porosity, water retention, and surface area to volume ratios appear to be the crucial properties of the supports used and these features interact with the branching mechanism of fungi. The most interesting concept relating those variables is the notion of steric hindrances of fungal growth within interstitial spaces. However, a great deal of work is necessary in order to link the heterogeneity of SSF systems with attractive high enzyme yields. Comparison of enzyme production between SSF and SmF systems is useful to derive some general working rules, such as the need to have solid supports with high porosity, water retention and surface area to volume ratios. There is also a need to select for organisms that produce at least ten times more enzyme units per gram of support as compared to their yields in units per milliliter in submerged fermentations expressed.

Research is needed to improve SSF systems in order to profit from their specific advantages over SmF systems, particularly in areas such as production of fine chemicals using artificial polymers and automated reactor designs. However, it is encouraging to note that at least a small part of the world enzyme market is already supplied by SSF systems.

ACKNOWLEDGEMENTS

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GLOSSARY OF TERMS

A, area of a single particle of a solid support (cm^2);
A_T, area of all the particles of a solid support (cm^2);
d_0, average diameter of hyphae (μm);
D, average diameter of solid particles in the support (cm);
E, enzyme concentration ($\mathrm{U/cm}^3$);
E_0, initial enzyme concentration ($\mathrm{U/cm}^3$);

E^L, volume enzyme concentration per unit volume of liquid fermentation broth (U/mL);
E^R, volume enzyme concentration per unit volume of the reactor (U/cm^3);
E^S, solid enzyme concentration on the support (U/g);
r^m, average surface area to volume ratio, for individual particles (cm^{-1});
r^M, average surface area to volume ratio, within the reactor volume (cm^{-1});
L_{av}, average length of hyphal distal segments (μm);
Q_X, amount of biomass in a given sample (g);
SmF, submerged fermentation;
SSF, solid-state fermentation;
S_0, initial substrate concentration;
k, secondary rate of enzyme change (U/gh);
K_{GI}, inhibition constant affecting, μ_M (g/l);
K_{GM}, saturation constant affecting, μ_M (g/l);
K_L, saturation constant affecting hyphal elongation (μm);
K_{XS}, saturation constant affecting maximal biomass level (g/l);
U_M, maximal rate of hyphal elongation (μm/h);
V, average volume of individual support particles (cm^3);
V_T, reactor volume (cm^3);
X_0, initial biomass concentration (g/l);
X^A, average biomass concentration per unit surface for a given S_0 value (g/cm^2);
X_M^A, maximal level of surface biomass concentration for a given value of S_0 (g/cm^2);
X_M^{A0}, maximal level of surface biomass concentration for a all the values of S_0 (g/cm^2);
X_M^{A0}, maximal value of the average biomass concentration per unit surface for all S_0 values (g/cm^2);
X^L, liquid biomass concentration in the fermentation broth (g/cm^3);
X_M^L, maximal value of liquid biomass concentration in the fermentation broth (g/cm^3);
X^R, volumetric biomass concentration in the reactor (g/cm^3);
X_M^R, maximal value of biomass volumetric concentration in the reactor (g/cm^3);
X^S, solid biomass concentration on the solid support (g/g);
X_M^S, maximal level of solid biomass concentration on the solid support (g/g);
$Y_{E/X}$, enzyme yield per unit of biomass (U/g);
α, porosity of solid support (dimensionless);
β, aspect of cylindrical objects (dimensionless);
γ, ratio between the primary and secondary rate of change of enzyme (dimensionless);
μ_M, maximal specific growth rate of biomass when $X \ll X_M$ for a given value of S_0;
μ_{GM}, maximal specific growth rate of biomass when $X \ll X_M$ for all the values of S_0;
ρ_S^m, microscopic density of solid support (g/cm^3);
ρ_S^M, macroscopic (apparent) or packing density of solid support (g/cm^3);
ξ, relative biomass concentration (X/X_M);
ξ_0, initial value of relative biomass concentration (X/X_M);
ω, water content of solid support (g_{H20}/g_S).

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Commercial Importance of Some Fungal Enzymes

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1 INTRODUCTION

Catalytic activity of enzymes has been known since the beginning of human civilization with the fermentation of sugar to ethanol by yeasts, a reaction that forms the basis of beer and wine manufacture. However, in the past half century, our increasing understanding of molecular structure and function has enabled us to design processes that utilize the enormous capacity provided by these naturally evolved catalytic systems. The estimated value of the world enzyme market is around US $1.3 billion and is forecasted to grow to almost US $2 billion by 2005. The main industries, which utilize enzymes, are detergents (34%), textiles (11%), starch (12%), baking (5%), animal feed (7%), beverages and brewing (7%), dairy processes (14%), and other uses (9%) (Figure 1). The major producers of commercial enzymes are Novo-Nordisk (Denmark) and Gist Brocades (Belgium), who cover about 40 and 20% of the market, respectively. Other companies such as Rohm (Germany), Miles (United States), and Hansens (The Netherlands) also have a significant market share. The current world market for industrial enzymes is over US $650 million per year (Waites et al. 2001).

At present more than 2000 different enzymes have been isolated and characterized and the structure of about 1300 different proteins has been elucidated. However, only a limited number of known enzymes are commercially exploited and more than 75% of industrial enzymes are hydrolases. Although the industrial and commercial application of biocatalysts is responsible for products worth billions of dollars, there is good reason to think that we have barely scratched the surface of the potential uses of enzymes. This chapter provides a brief overview of the current diverse enzyme-based industrial processes and the possible areas of enzyme technology that may be developed in the near future.

2 AMYLASES

Amylases are among the most important industrial enzymes in commercial biotechnology (Pandey et al. 2000). Amylases hydrolyze starch molecules to diverse products such as dextrin, and progressively smaller polymers of glucose units. In 1950, Mybark and Neumuler classified amylases into two categories: (a) endoamylases and (b) exoamylases. “Endoamylases” split linkages in starch molecules in a random manner. This action results in linear and branched oligosaccharides of various chain lengths. “Exoamylases” hydrolyze starch from the non-reducing end, giving a series of successively shorter products. The primary enzymes involved in starch degradation are α- and β-amylases. α-Amylases (α-1-4 glucan glucohydrolase) are endoamylases and hydrolyze α-(1-4) glycosidic linkages in starch randomly and are capable of bypassing the branch points. β-Amylases are exoacting enzymes and usually occur in plants and some microbes. These enzymes hydrolyze the β-(1-4) linkages from the nonreducing end of the starch molecule and bypass the branch points of substrates such as amylopectin or glycogen. The end products of hydrolysis are maltose and high molecular weight β-limit dextrins. In addition to these there are various other enzymes, which are involved in starch degradation, i.e., glucoamylase, α-glucosidase, isoamylase, pullalanase, and cyclodextrin glycotransferase.

The first industrial use of a fungal enzyme was an amylase from *Aspergillus oryzae* in 1894. Since then there have been many reports on amylase production from both mesophilic and thermophilic fungi (Vihinen and Mantsala 1989; Fogarty and Kelly 1990), and amylase production has been reported in species of the genera *Aspergillus*, *Penicillum*, *Rhizopus*, *Mucor*, *Humicola*, and *Thermomonospora*.

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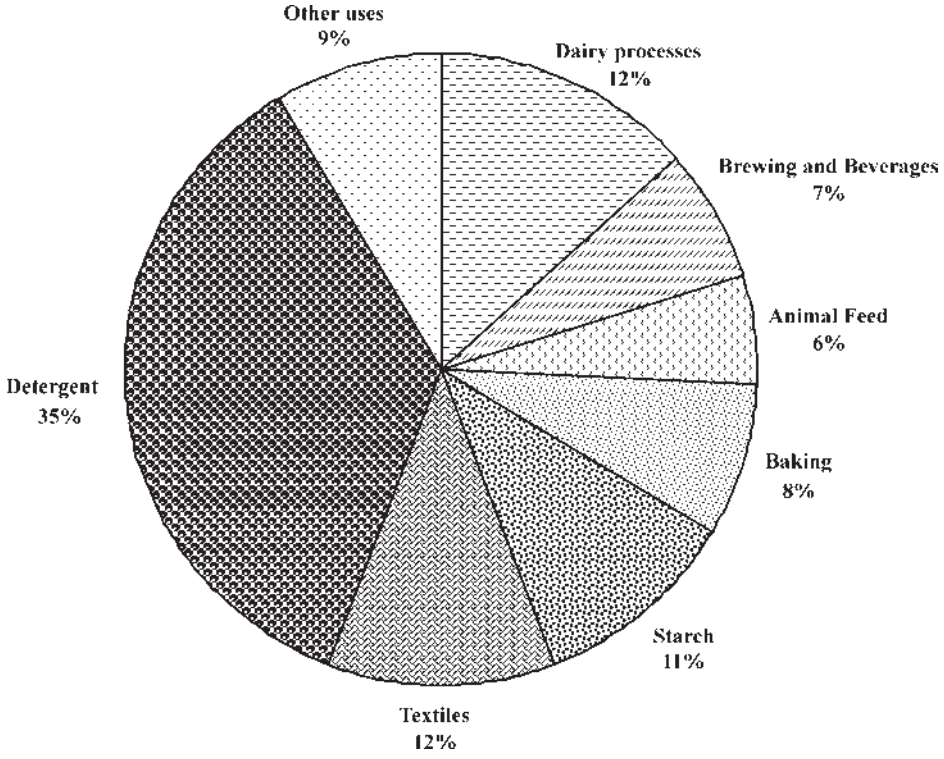

Figure 1 Application of bulk microbial enzymes.

2.1 Applications of Amylases

2.1.1 Food and Dairy Industry

Enzymes such as malt and microbial α-amylases have been used for bread making for decades. α-Amylases derived from fungi have since replaced the malt-derived enzymes, as malt α-amylases alter the color of the bread and have higher protease content. Fungal α-amylase has been permitted as a bread additive in the United States since 1955 and in United Kingdom since 1963 (Pritchard 1992). Amylases are also found to have some effects on pasta quality and in noodle production. The major markets for starch hydrolysates are in syrups and sweeteners, and they are used in the beverage industry as sweetener for soft drinks due to their high sweetening property. The process requires the use of a highly thermostable α-amylase for starch liquefaction and these enzymes are applied as a mixture with other α-amylases for ethanol fermentation.

2.1.2 Textile and Paper Industry

A good desizing of starch-sized textiles is achieved by the application of α-amylases. α-Amylases selectively remove the size and do not attack the textile fibers. The α-amylases randomly cleave the starch into dextrins that are water-soluble and can be removed by washing. Therefore use of amylases in the pulp and paper industry is in the modification of starch for coated paper. As for textiles, sizing of paper is performed to protect the paper against mechanical damage during processing and also improves the quality of the finished paper. The viscosity of natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with α-amylases. A number of amylases obtained from fungi are used in paper industry and these include Amizyme® (PMP Fermentation Products, Peoria, USA), Termamyl®, Fungamyl®, BAN® (Novo-Nordisk, Denmark) and alpha-amylase G995® (Enzyme Biosystems, USA).

2.1.3 Detergent Industry

α-Amylases are used in laundry detergents (Kottwitz et al. 1994) and there is an increasing demand for α-amylases for automatic dishwashing detergents. Enzyme stability in the presence of oxidants in household detergents has been achieved by following the successful strategies used for other enzymes such as proteases. Two major detergent enzyme suppliers, Novo Nordisk and Genencore International, have recently used protein engineering to improve the bleach stability of amylases (Tierny et al. 1995). They independently replaced the oxidation-sensitive amino acids by other amino acids and have introduced these new products under the trade names Purafect OxAm® and Duramyl®.

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2.1.4 Medical

There are several processes in the medicinal areas that involve the application of amylases. Sutton et al. (1999) assessed 13 compounds, including amylase, for serum evaluation. Menzel et al. (1998) have developed biosensors with an electrolyte isolator semiconductor capacitor (EIS-CAP) transducer for process monitoring. α-Amylase has been used to prepare a hybrid membrane using chitosan as a dispersant in the sol-gel process by Cho et al. (1997). α-Amylase has also been used as an enzyme thermistor for the biochemical analysis of cyclodextrins (Kolb et al. 1996).

3 PROTEASES

Proteases are complex group of enzymes collectively known as peptidyl-peptide hydrolase (EC 3.4.21-24 and 99). These may include proteinases and peptidases that hydrolyze the peptide bonds in protein molecules. Microbial proteases have to a certain extent replaced the traditional proteases from animal and plant origin, and in addition, have found further applications due to their special properties. Fungi are known to produce three different types of proteases, namely acid, neutral, and alkaline.

Many mesophilic and thermophilic fungi have been exploited for the release of proteolytic enzymes including species of the genera *Aspergillus*, *Mucor*, *Penicillum*, *Rhizopus*, and *Sporotrichum*.

3.1 Applications of Proteases

Proteases account for a major share of global enzyme market. They have been used for the production of value-added products in diverse fields and in various industries.

3.1.1 Detergent Industry

The largest application of proteases is in the formulation of household laundry detergents. Proteases hydrolyze coagulated proteins so that the soluble degradation products are easily rinsed off the fabric during washing. There are many parameters that are considered in the selection of a good detergent protease such as compatibility with other detergent components such as surfactants, good activity at the relevant washing temperature and pH, compatibility with the ionic strength of the detergent (solution), stain degradation and/or removal potential, stability and shelf life.

3.1.2 Food and Dairy

Fungal acid proteases are used as substitutes for papain, rennin, and pepsin. Among the fungi, species of *Aspergillus* and *Mucor* are particularly important sources of proteases for industry. *Aspergillus flavus*, *A. niger*, *A. oryzae*, *Rhizomucor pusillus*, *R. meihei*, and *Rhizopus* species are used in the preparation of the oriental foods such as Tempeh and Koji and in cheese making as substitutes for natural rennet. *R. pusillus*, *R. meihei*, *Penicillium roueforti*, *P. camemberti*, and *Endothia parasitica* are important sources of milk-clotting enzymes (Sternberg 1976). Proteases are also used for the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates are generally used in infant food formulations, specific therapeutic dietary products, and for the fortification of fruit juices and soft drinks. Cheese whey is an abundant liquid by-product of the dairy industry and there is an estimated world production of 145 million tones per annum. Parera et al. (1993) have used alkaline protease for the production of whey hydrolysate from the cheese whey although rennet is generally the enzyme of choice for cheese industries.

3.1.3 Leather Industry

Alkaline proteases with elastolytic and keratinolytic activity have been used in leather processing, especially for the dehairing and debating of skins and hides. The enzymatic process is easy to control, less time consuming, and also helps in waste management, and is therefore eco-friendly. In addition, enzymatic treatment destroys undesirable pigments and increases the skin area, thereby producing clean hide. Bating is traditionally an enzymatic process involving pancreatic proteases but the use of fungal alkaline protease has recently become popular (Malathi and Chakraborty 1991).

3.1.4 Medical Industry

Alkaline proteases have also been used for developing products of medical use, such as for the treatment of burns and purulent wounds, carbuncles, furuncles, and deep abscesses. *Aspergillus niger* LCF9 alkaline protease has a high collagenolytic activity and is being used for therapeutic applications in the preparation of slow-release dosage fonns (Kumar and Takagi 1999).

3.1.5 Other Industrial Uses

Proteases are known for their catalytic use in synthetic chemistry (Zaks 1991). Alkaline protease is used in peptide synthesis and also in the resolution of the racemates of amino acids, where Kawashiro et al. (1997) have reported the use of alkaline protease in enantioselectivity. Proteases can solubilize proteinaceous wastes and so lower the BOD of waste systems. Alkaline protease has opened up a new era in the management of wastes from various food processing industries and household activities, and Dalev (1994) has used alkaline protease for the management of waste feathers from poultry slaughterhouses.

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4 LIPASE

Lipases are today one of the most important class of industrial enzymes in use due to their versatile catalytic behavior (Jaeger and Reetz 1998; Kazlauskas and Bornscheuer 1998; Saxena et al. 1999).

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) belong to the class of serine hydrolases that catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol, and fatty acids. Lipases have been extensively studied both for academic and industrial applications due to their multiplicity, specificity and stability and their chemo-, regio-, and enantioselective nature for various enzymatic reactions.

Fungal lipases have been widely studied since the 1950s and Lawrence (1967), Pandey et al. (1999), and Saxena et al. (1999) have provided comprehensive reviews. The major producers of commercial lipases are *A. niger*, *Candida cylindracea*, *Humicola lanuginosa*, *M. meihei*, *R. arrhizus*, *R. delmer*, *R. japonicus*, *R. niveus*, and *R. oryzae* (Godtfredsen 1990).

4.1 Applications of Lipases

4.1.1 Food Industry

The biolytic release of short- and medium-chain fatty acids (C: 4 to C: 10) from milk fat triglycerides is responsible for the development of desirable flavors in dairy products (Kilara 1985). A whole range of fungal lipase preparations have been developed for the cheese manufacturing industry, such as those from *M. meihei*, *A. niger*, and *A. oryzae*. A range of good quality cheeses has been produced by using individual lipases or mixture of several preparations (Falch 1991). Lipases are also used for cheese made from ewe's or goat' milk, and the resulting cheese is called enzyme-modified cheese (EMC).

4.1.2 Cosmetics and Perfume Industry

The main reason for screening lipases for use in the cosmetic and perfume industry has been their activity as surfactants and in aroma production. Monoacylglycerols and diacylglycerols, prepared by the lipase-catalyzed esterification of glycerol, are useful as surfactants in cosmetics (Berger and Schneider 1991). Izumi et al. (1997) performed the transesterification of 3,7-dimethyl-4,7-octadien-1-ol with lipases from various microbial sources to prepare rose oxide, which is an important fragrance ingredient in the perfume industry. Miyamoto et al. (1995) obtained a patent for the use of lipases in the production of glycerine mixtures that were used as an external preparation or for direct application to skin. Nippon-Oil & Fats (1986) also obtained a patent for the preparation of propyl-eneglycerol mono-fatty acid ester in the presence of lipase. This ester has uses as an emulsifier and a pearling agent in both cosmetics and foods.

4.1.3 Environmental Management

Bioremediation for waste disposal is a new development of lipase biotechnology. Oil spills resulting from rigging and refining, oil-wet night soils and shore sand, and lipid-tinged wastes in lipid processing factories and restaurants can all be treated by the use of lipases of different origins (Salleh et al. 1993; Sarada and Joseph 1993). The increasing use of lipases in bioremediation has achieved greater importance with its successful application in the upgrading of waste fat (Salleh et al. 1993).

4.1.4 Leather Industry

Leather processing involves the removal of subcutaneous fat, dehairing, and stuffing. An enzyme preparation that contained lipases in combination with other hydrolytic enzymes such as proteases would provide a new development in leather processing. A new enzymic process to produce hides and skins ready for tanning, involved soaking, washing, dehairing, and bathing in aqueous baths, where each bath had a pH of 8–13 and contained an alkalophilic lipase (Macrae and Hammond 1995).

4.1.5 Detergent Industry

Lipases have been used in the formulations for cleaning drains clogged with food, and/or nonfood plant material containing deposits. Removal of oil and fat deposits by lipase is an attractive prospect due to its activity under washing conditions. Lipolase, introduced by Novo, was developed by the overproduction of lipase by expression of *H. lanuginosa* lipase gene in *A. oryzae*.

4.1.6 Medical Industry

Conventional chemical synthesis of drugs containing a chiral center generally results in equal mixtures of enantiomers (Reddy 1992). Recently, many studies have shown that the desired therapeutic activity of racemic drugs is usually located mainly in one of the enantiomers, and that the other enantiomers can interact with different receptor sites, which may cause unwanted side effects. Cui et al. (1997) reported lipase-catalyzed esterification in organic solvent to resolve racemic naproxen. Naproxen is a nonsterol anti-inflammatory drug whose (S)-enantiomer is 28-times more active than that the corresponding (R)-enantiomer. Akita et al. (1997) used enzymic hydrolysis in organic solvents with immobilized lipases for the kinetic resolution of water-insoluble a-acyloxy esters to produce chiral intermediates for the synthesis of the calcium antagonist diltiazem hydrochloride and the antibiotic (®)-indolmycin. *A. niger* and *Penicillium urticae* lipases were highly enantiospecific ($E^{-}100$), and frequently cleaved undesired enantiomers; *Candida rugosa* lipase, however, was highly (S)-specific (Jansen et al. 1996). Hernaiz et al. (1997) reported two stereoselective isoenzymes, A and B from *C. rugosa*. Although both isoenzymes esterified (S)-ibuprofen, lipase-B was more stereoselective. Lipases

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from *A. carneus* and *Aspergillus terreus* show both chemo- and regiospecificity in the hydrolysis of peracetates of pharmaceutically important polyphenolic compounds (Parmar et al. 1998a,b).

4.1.7 Oleochemical Industry

There is a considerable scope for the application of lipases in oleo-chemical industry, as these may save energy and minimize thermal degradation during hydrolysis, glycerolysis, and alcoholysis. The Miyoshi Oil and Fat Co. have reported the commercial use of *C. cylindracea* lipase in the production of soap (McNeill and Yamane 1991). Another potential application of lipases, particularly those of fungal origin is in the synthesis of polyunsaturated fatty acids (PUFA) such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) that are beneficial in cardiovascular and inflammatory diseases. *R. meihei* lipase increases the proportion of EPA and DHA in soyabean oil to a final concentration of 10.5–34.7% (Huong and Akoh 1994) and in Sardine oil upto 70% in the presence of ethylene glycol (Hosokawa et al. 1995). Another potential application of microbial lipases is in the synthesis of "zero-trans" margarines (Marangoni and Rousseau 1995) as trans isomers are reported to contribute to heart disease. There are many other important areas in which lipases have an important role and these are listed in Table 1.

5 XYLANASE

Xylanolytic enzymes act cooperatively to degrade xylan to its constituent simple sugars. Xylanases have been reported to occur in marine and terrestrial fungi (saprophytes, phytopathogens, and mycorrhiza), protozoa, ruminant bacteria, marine algae, snails, crustaceans and insects and in germinating seeds of terrestrial plants (Sunna and Antranikian 1997; Kuhad et al. 1997).

There has been an increasing interest in the use of fungi because of their ability to produce high levels of xylanolytic enzymes and as a result several groups of fungi have been screened for xylanase production. Mesophilic xylan degrading fungi include *A. niger*, *A. fumigatus*, *A. terreus*, *Neurospora crassa*, *Trichoderma reesei*, and *C. tropicalis*. Xylan degrading thermophilic fungi includes *H. lanuginose*, *Thermoascus aurantiacus*, and *Melanocarpus albomyces*.

5.1 Applications of Xylanases

Xylan-metabolizing micro-organisms and their enzyme systems have become important tools in developing economically and ecologically beneficial processes for the use of the most abundant renewable polysaccharides.

5.1.1 Bioconversion

Xylan-rich liquors may be used by xylanolytic microbes to produce a mixture of fermentable sugars (Biely 1985). Xylose obtained from the hydrolysis of xylan has been used as a substrate for the production of ethanol, xylitol, xylonic acid, and other chemicals (Horitzu et al. 1992). Pentose produced by the hydrolysis of xylans can also be used as substrates in the production of protein rich biomass (e.g., by *C. utilis* in Single Cell Protein) and in the saccharification of agricultural and forestry wastes and residues for fermentation to fuels.

Table 1 Important areas of industrial applications of fungal lipases

Industry	Effect	Product
Bakery	Flavor improvement and increased shelf life	Bakery products
Beverages	Improved aroma	Beverages
Chemicals	Enantioselectivity	Chiral building blocks and chemicals
Cleaning	Synthesis	Chemicals
	Hydrolysis	Removal of cleaning agents such as surfactants
Cosmetics	Synthesis	Emulsifiers, moisturizing agents
Dairy	Hydrolysis of milk fat	Flavor agents
	Cheese ripening	Cheese
	Modification of butter fat	Butter
Fats and oils	*Trans*-esterification	Cocoa butter, margarine
	Hydrolysis	Fatty acids, glycerol, mono- and diglycerides
Food dressing	Quality improvement	Mayonnaise, dressings, and whippings
Health food	*Trans*-esterification	Health food
Leather	Hydrolysis	Leather products
Meat and fish	Flavor development and fat removal	Meat and fish products
Paper	Hydrolysis	Paper products
Pharmaceuticals	*Trans*-esterification	Speciality lipids
	Hydrolysis	Digestive aids

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5.1.2 Food and Feed Industry

Xylanolytic enzymes, together with cellulases and pectinases, are widely used for the clarification of juices and wines. These enzymes also liquefy fruits and vegetables in the production of purees (Biely 1985; Lekha and Lonsane 1997). Fungal xylanases have also been used to modify cereal flours to enhance their volume, textural, coloring, and flavoring properties. The other main applications of xylanases are in the saccharification of agricultural, industrial, and municipal wastes to provide sugar syrups for human or animal consumption, the production of modified xylanase as bulking agents and as food thickeners in food processing. In addition, xylanases are used as a feed supplement to improve the nutritional value of agricultural silage and poultry diets. The incorporation of xylanase into a rye-based diet for broiler chickens improved the weight of chicks and their feed conversion efficiency.

5.1.3 Textile Industry

Xylanolytic and pectinolytic enzymes can be used together for the accelerated retting of hemp, flax, jute, sisal, and bast fiber and for the production of viscose rayon, cellulose esters, and cellulose ethers.

5.1.4 Pulp and Paper Industry

Xylanases are used for the enzymatic pre-bleaching of pulp for paper manufacture, and are also used for debarking, beating, pulp fiber refining, and in the production of dissolving pulps. The major aim of enzymatic debarking and pulp refining is the reduction of energy demands during the mechanical processes. The methods require cellulase-free, thermostable and alkalostable xylanases as the industrial process takes place at high temperature and under highly alkaline conditions. The partial hydrolysis of hemicellulose bound to lignin in pulps can improve the subsequent removal of lignin during chlorine treatment. Therefore the prior treatment of pulps with xylanases can reduce the amount of chlorine required and improve both the economics and environment friendliness of the process. Pretreatment of pulps with xylanases prior to bleaching also reduces the bleached chemical requirements and results in greater brightness. Most of the studies on the effect of xylanases in pulp bleaching have been conducted with fungal enzyme preparations (Daneault et al. 1994; Dasilva et al. 1994). Jager et al. (1992) reported a 30% reduction of chlorine and 38% reduction in hypochlorite consumption when beechwood kraft pulp was enzymatically pretreated with xylanase of *Thermomyces lanuginosus*. A thermophilic xylanase from *Sporotrichum dimorphosum* has been used to lower the lignin content of unbleached softwood and hardwood kraft pulp (Chauvet et al. 1987). Pretreatment of pulp with a xylanase from *Saccharomonospora viridis* showed that paper produced from enzyme treated pulp showed a slight decrease in interfiber bond strength (Roberts et al. 1990).

6 TANNASE

The enzyme tannase (tannin acyl hydrolyase, EC 3.1.1.20) catalyzes the hydrolysis of ester and depside bonds in hydrolyzable tannins such as tannic acid, thereby releasing glucose and gallic acid. Tannases are also able to carry out the reverse reaction of synthesis in organic solvents in a similar fashion to lipases.

6.1 Applications of Tannases

6.1.1 Food and Feed Industry

Tannase catalyzes the de-esterification of tea-leaf components leading to enhancement of the natural level of gallic acid and epicatechin, and this in turn favors the formation of large amounts of epitheaflavic acid. This compound is responsible for the bright reddish-black tea-like color and has very good cold-water solubility. Further deesterification of green tea leaf constituents prevents the formation of any gallated tea oxidation products.

Massechelin and Batum (1981) reported that tannase produced by a strain of *A. flavus* significantly reduced chill haze formation in beer. Yamada and Tanaka (1972) had earlier reported the use of tannase in the treatment of grape juice and wine. The enzyme hydrolyzed chlorogenic acid to caffeic acid and quinic acid, compounds that favorably influenced the taste. Tannase has also been used with laccase to treat grape juice and grape musts, in order to remove phenolic substances and stabilize the beverage (Canteralli et al. 1989).

Tannase has been employed in the biocatalytic conversion of glucose to gallic acid and also to reduce the hydrolyzable tannin levels in poultry feeds. These reactions have allowed the use of cheaper tannin containing components in animal feed. Tannase has potential applications in the treatment of olive oil waste water and the partial decolorization by enrichment cultures due to its capability to hydrolyze tannic acid and gallic acid esters.

6.1.2 Chemical Industry

Tannase produced from *A. niger* has been used in the production of gallic acid (98% purified; yield of 9.75%) from gallotannins of *Rhus coriaria* (Pourrat et al. 1985). It has also found applications in the manufacture of printing inks, dye stuffs, and as an ingredient of photographic developer. A major use of gallic acid is in the manufacture of an antibacterial drug, trimethoprim. Tannase also catalyzes the synthesis and trans-esterification of gallic acid esters such as propyl gallate, a widely used food preservative (Macrae 1983).

7 PHYTASE

Phytase is an enzyme that hydrolyzes phytic acid to myo-inositol and phosphoric acid in a stepwise manner forming

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myoinositol phosphate intermediates. Phytase has been the subject of research for 87 years from its discovery until its commercialization in Europe in 1993–1994 by Gist-Brocades. There are two types of phytases: (a) 3-phytase (myo-inositol-hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8), which hydrolyzes the ester bond at the 3-position of the myo-inositol hexakisphosphate to D-myo-inositol 1,2,4,5,6-pentakisphosphate and orthophosphate and (b) 6-phytase (myo-inositol-hexakisphosphate 6-phospho-hydrolase, EC 3.1.3.26), which hydrolyzes the 6-position of the myo-inositol hexakisphosphate to *d*-myo-inositol 1,2,3,4,5-pentakisphosphate and orthophosphate.

Phytase activity has been most detected frequently in fungi. Over 200 fungal isolates of the genera *Aspergillus*, *Penicillum*, *Mucor*, and *Rhizopus* have been tested for phytase production and the highest phytase production has been reported from *Aspergillus* species.

7.1 Applications of Phytases

Phytases have two major applications: for the removal of phytic acid in feed and food industries, and for in the preparation of special myo-inositol phosphates as tools for biochemical investigation.

7.1.1 Food and Feed Industry

The availability of phosphorus can be improved by adding microbial phytase to feeds or by using phytase rich cereal diets. The enzyme minimizes the need for supplementation with inorganic phosphorus, due to its ability to improve the utilization of organic phosphorus in poultry, and thus markedly reduces the excretion of phosphorus in manure (Mohanna and Nys 1999). Degradation of myo-inositol phosphates yield myo-inositol, which is an important growth factor, and so can improve the digestibility and nutritive value of feeds. Alko Co. (Finland), Altech (USA), and BASF (USA) have all started industrial scale production of phytase (Trade names: Finase, Allzyme phytase, and Natuphos). Canola meal, used as a feedstuff for livestock and fowl has been successfully treated with phytase by *A. niger* in solid-state fermentation (Ebune et al. 1995). Similarly *A. carbonarious* phytases (Al-Asheh and Duvjnak 1995a,b) have been used to reduce the phytic acid content in rape and canola meals in solid-state fermentation processes. Research on enzymic cocktails consisting of phytase A and B, pectinase and citric acid, and cocktails in either soluble or intracellular form (fungal mycelium) can enhance dephosphorylation and influence phytate conversion rate (Zyta 2001).

7.1.2 Chemical Industry

Inositol phosphates and phospholipids are important in both transmembrane signaling and in the mobilization of calcium from intracellular reserves. There has been, therefore an increasing interest in the formulation of various inositol phosphate preparations. Siren (1986) described the preparation of D-myoinositol 1,2,6-trisphosphate, D-myoinositol 1,2,5-trisphosphate, L-myoinositol 1,3,4-trisphosphate, and myoinositol 1,2,3-trisphosphate by enzymatic hydrolysis of phytic acid using *Saccharomyces cerevisiae* phytase. Inositol phosphate derivatives can be used as enzyme stabilizers, as enzyme substrates for metabolic investigation, as enzyme inhibitors and have pharmaceutical uses, and as chiral building blocks (Laumen and Ghisalba 1994).

7.1.3 Pollution Abatement

One of the major driving forces in the development of phytase as a product is the awareness of the environmental effects of the release of concentrated pollutants. When microbial phytase was added to low-P diets fed broilers, the availability of phosphorus increased to 60% and the amount of phosphorus in the bird droppings decreased by 50%. There may therefore be potential benefits of phytase in commercial diets and reducing phosphate pollution in soil and water (Simon et al. 1990).

8 ENZYME IMMOBILIZATION

Immobilization refers to the localization or confinement of enzymes during a process, which allows the enzyme to be readily separated from the product and substrate for reuse. The advantages of immobilization of enzymes are (a) multiple or repetitive use of a given batch of enzyme, (b) better process control, (c) enhanced stability, (d) enzyme free products, (e) long half life and predictable decay rates, and (f) good models for the study of in vivo enzyme kinetics.

The greatest economic returns from immobilization are achieved with expensive enzymes, as there is a definitive cost associated with immobilizing process. Economics of immobilized enzyme systems require the determination of the cost of the individual components vs. value of the system's performance.

8.1 Techniques for Immobilizing Enzymes

There are five markedly different techniques for immobilizing enzymes.

8.1.1 Adsorption of the Enzyme on a Carrier Surface

This procedure is the simple and most economic. The surface activity of the support acts in concert with a functional moiety or characteristic group on the surface of the enzyme protein, resulting in the immobilization of the enzyme. Bonding between enzyme protein and the carrier may be ionic, hydrogen, covalent, hydrophobic, or by a combination of these.

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8.1.2 Covalent Coupling of the Enzyme to the Carrier Surface

Covalent attachment to surfaces generally offers several advantages for an immobilized enzyme system including its use under a wide range of pH conditions, ionic strengths, and uncontrolled variable conditions. As covalent coupling may be directed to a specific group (amine, hydroxyl, tyrosyl, etc.) on the surface of the enzyme, the active site of the enzyme can be avoided by the selection of a group on the surface of the enzyme protein that is not a part of the site. Covalent coupling is the preferred immobilization method for enzymes that contain polymeric units or prosthetic groups. Covalent techniques may be tailored to give enzyme composites suitable for specific surfaces or environments.

8.1.3 Crosslinking Between Enzyme Molecules (Copolymerization)

Crosslinking between enzyme molecules and other monomers is essential to obtain an insoluble preparation that can be readily handled or manipulated in a continuous reactor. The enzyme activity of immobilized preparations crosslinked with multifunctional reagents is dependent upon the degree of crosslinking with high crosslinking giving to lower activities and vice versa.

8.1.4 Entrapment of the Enzyme in a Matrix

This procedure involves the physical confinement of an enzyme in some form of matrix. It is distinguished from crosslinking and copolymerization in that the enzyme is not chemically part of the matrix. The most frequently employed entrapment matrix is polyacrylamide, but starch, collagen, and silicon rubber have also been used. Due to the major limitations of enzyme leaching and pore diffusion limitations, it has not achieved a great deal of success in industrial processes.

8.1.5 Encapsulation in a Membrane Structure

This method can be distinguished from entrapment by the fact that the enzyme is separated from the bulk solution by encapsulation in a membrane, while entrapment involves a lattice-immobilized enzyme. Membrane encapsulation involves for larger quantities of enzyme per unit volume to be immobilized than any other procedure.

8.2 Overall

Although each immobilization technique is unique, they are not mutually exclusive. Adsorption on a carrier surface, either intentionally or unintentionally, will involve some degree of crosslinking between enzyme molecules. Covalent coupling to a carrier surface usually involves adsorption of enzymes on that surface and crosslinking between enzyme molecules. Entrapment of an enzyme in a matrix may involve adsorption, covalent coupling to the surface or crosslinking between the enzyme molecules. Encapsulation or confinement of an enzyme solution within a membrane structure minimizes the contamination by these mixed immobilization effects, however, some adsorption on the membrane surface and some crosslinking of the molecules will occur.

8.3 Immobilization of α-Amylase

Alpha-amylase from *A. oryzae* has been immobilized on porous glass and silochrome by glutaraldehyde crosslinking. A new method for the immobilization of α-amylase by UV curing on piezoelectric crystal has also been described (He et al. 2000). Under certain experimental conditions, the immobilized enzyme can be reused 50 times. Composite hydrogel membranes of crosslinked poly (*N*-isopropyl-acrylamide-co-*N*-acryloxysuccinimide-co-2-hydroxyet ethyl methacrylate) [P (NIPAAm-NAS-HEMA)], together with starch, on nonwoven polyester has also been used to immobilize α-amylase (Sun et al. 1999).

8.4 Immobilization of Protease

Protease from *R. miehei* and other milk-clotting enzymes have been immobilized on porous alkylamide glass (Cheryan et al. 1975). Rennet from *R. pusillus* has been immobilized on cotton cloth using paraffin wax and this has proved to be a rapid, cheap, and simple method capable of producing good quality, reusable enzyme (Mashley 1987).

8.5 Immobilization of Lipase

Immobilization of lipases by adsorption increases the surface area and also avoids the lyophilization of the lipase. Celite, Amberlite, and accurel are the most commonly used supports. Immobilization of lipases by entrapment, especially in hydrophobic sol-gels, has also been tried (Kawakami and Yoshida 1996) and lipases entrapped in these gels can have a 100-fold increased activity in organic solvents (Reetz et al. 1997). The most common method of covalent immobilization is by crosslinking the adsorbed lipases with gluteraldehyde and this increases their operation and thermal stability. Lipase from *C. rugosa* can be immobilized by adsorption onto polypropylene powder EP100 and used in the chiral synthesis of ibuprofen and *trans*-2-phenyl-1-cyclohexanol esters (Lopez et al. 2002).

8.6 Immobilization of Xylanase

In contrast to many hydrolases, xylanases have not been commonly immobilized as the enzyme acts on insoluble substrates and this limits its re-usability. The noncovalent

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immobilization of a commercial preparation of xylanase from *A. niger* was carried out on the reversibly soluble–insoluble enteric polymer Eudragit (TM) L-100 (Sardar et al. 2000). Immobilization of the xylanase activity by adsorption was simultaneously accompanied by removal of cellulase activity since the latter did not bind to the polymer. The soluble enzyme derivative may be useful for treatment of paper pulp bleaching, while the immobilized xylanase retained 60% of its activity on xylan (Sardar et al. 2000). Xylanase from two isolates of *Aspergillus* was noncovalently immobilized on Eudragit S-100 for saccharification. On immobilization, the optimum activity temperature increased between 50 and 60°C, as compared to 50°C in the case of free enzymes. Immobilization increased enzyme stability mainly by decreasing the temperature sensitivity to the inactivation reaction. Enzymatic saccharification of xylan and wheat bran has been improved by xylanase immobilization with immobilized xylanase from both the strains producing three times more sugar as compared to free xylanase. Immobilized xylanase has been recycled three times in repeated batch saccharification studies without loss of enzymatic activity (Gawande and Kamat 1998).

8.7 Immobilization of Tannase

The first report on the immobilization of tannase was by covalent attachment to alkylamine porous silica activated with gluteraldehyde (Wheetal and Datar 1974). The pH for optimum tannase activity increased slightly and thermal stability was increased after immobilization. Another method of immobilization used is covalent binding of tannase by its glycosidic part to an insoluble support (Nicholas et al. 1997). Tannase from *A. oryzae* has been immobilized on various carriers including chitosan, chitin, Dowex 50 W, and DEAE-sephadex A-25 by different methods. The enzyme immobilized on chitosan with a bifunctional agent (gluteraldehyde) had the highest activity. The catalytic properties and stability of the immobilized tannase changed as the temperature optimum shifted from 40 to 55°C. The optimum pH also fell and the immobilized enzyme retained 85% activity even after being used 17 times (Abdel-Naby et al. 1999).

8.8 Immobilization of Phytase

Phytase from *A. ficuum* has been covalently immobilized into polyurethane foams at very high enzyme loadings (0.2 g protein/g dry foam) (Bakker et al. 2000). *A. ficuum* phytase has also been covalently immobilized on Fractogel TSK HW-75 containing 2-oxy-l-alkylpyridinium salts. The immobilized phytase was able to hydrolyze myo-inositol hexa-, penta-, tetra-, tri-, and diphosphates. When the substrate solution was recirculated for 5 h in a bioreactor about 50% inorganic orthophosphate was released and myo-inositol-diphosphate and mono-phosphate were the only remaining products (Ullah and Phillippy 1988).

9 CONCLUSIONS

The market for industrial enzymes continues to grow steadily. Major reasons for this are improved production efficiency resulting in cheaper enzymes in new applications and in new enzymes from screening programs or in newly engineered properties in traditional enzymes. New applications are to be expected in the fields of textiles and new animal diets, such as for ruminants and fish feed, and in pulp and paper treatments. Tailoring enzymes for specific applications increase in the future due to continuously improving tools, greater understanding of structure–function relationships, and increased use of enzymes from exotic environments. Future technological improvements are to include today the use of enzymes as crystalline catalysts, the increased recycling of cofactors, and the engineering of enzymes to function in different solvents and under different conditions.

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23

Xylanases of Thermophilic Molds and Their Application Potential

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1 INTRODUCTION

The xylanolytic potential of thermophilic molds has attracted interest for decades because of their pivotal role in composting and degradation of agro-residues, mushroom production, and solid waste management. A spurt of interest in these enzymes in recent years is due to their application in various industrial sectors like, paper and pulp, animal-feed, food, wine, textile, and pharmaceuticals (Kuhad et al. 1997). Thus, their biotechnological applications would help in fulfilling the increased demand of food and fuel for the staggerdly and nonexponentially increasing population world-wide, besides, resulting in economically viable and eco-friendly technologies. Xylanase hydrolyses β-1,4 glycosidic linkages between D-xylanopyranose units of xylan, a heteropolysaccharide with substitutions like, acetylation at the C-2 or C-3 of the xylose units; α-1,2-linked glucuronic or 4-*O*-methylglucuronic acid groups; α-1,3-linked arabinofuranose units and ferulic or coumaric acids esterified to C-5 of arabinose (Coughlan 1992). Xylan, is a heterogeneous substrate that requires synergistic action of main-chain and side-chain cleaving enzymes for complete hydrolysis. The main-chain cleaving enzymes include: endo-xylanase (β-1,4-D-xylan xylanohydrolase, E.C.3.2.1.8), which is well characterised in *Chaetomium thermophile*, *Humicola grisea*, and *Talaromyces emersonii* (Ganju et al. 1989; Thakur et al. 1992; Tuohy et al. 1993); exoxylanase (β-1,4-Dxylan xylanohydrolase), whose existence has been reported in some thermophilic molds like, *Chaetomium thermophile* (Ganju et al. 1989), but is not a well-studied enzyme, and β-xylosidase (β-1,4-D-xylan xylanohydrolase, E.C.3.2.1.37), produced in appreciable amounts by *Aspergillus fumigatus*, *Humicola lanuginosa*, and *T. emersonii* (Anand and Vithayathil 1996; Kitpreechavanich 1984; Tuohy et al. 1993). The side-chain enzymes act cooperatively by removing side chain substituents prior to depolymerisation of the high molecular weight backbone by the main chain cleaving enzymes. These enzymes include: α-L-arabinofuranosidase (AF) (E.C. 3.2.1.55), well characterised in *Rhizomucor pusillus* and *T. emersonii* (Rahman et al. 2001; Tuohy et al. 1993); acetyl (xylan) esterase (E.C. 3.1.1.6), reported in *Melanocarpus albomyces* (Jain 1995) and α-D-glucuronidase (E.C. 3.2.1??), which is characterised in *Thermoascus aurantiacus* (Khandke et al. 1989c).

The hydrolysis products of xylan may be subsequently converted into liquid fuel, SCP, solvents, and other chemical products (Kuhad et al. 1997). The increased industrial demand for thermostable enzymes with high pH stability makes xylanases potential candidate for biotechnological applications. During the last years, unravelling the structure of these enzymes has been a priority so that further improvement in thermostability and activity can be achieved by site-directed mutagenesis, gene manipulation, and protein engineering. This chapter deals with various facets of xylanolytic enzyme system of thermophilic molds.

2 PRODUCTION OF XYLANASES

A number of thermophilic fungi are known to secrete xylanases utilizing agro-residues (Table 1). Among these, *Thermomyces lanuginosus* (formerly *H. lanuginosa*) is an effective xylanase producer in nature. Strain variability in production and complexity of the enzyme components has been reported in this and other thermophilic molds (Chadha et al. 1999; Puchart et al. 1999). Based on substrate utilization, the relative variation in xylanolytic ability followed the order: *Sporotrichum thermophile* > *Acremonium alabamensis* >

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Table 1 Production characteristics of xylanases of thermophilic molds

Organism	Mode of cultivation	Substrate	Temperature (°C)	pH	Time (h)	Maximum production (IU /ml)	Reference
A. alabamensis	LSF	Paddy straw	45	7.0	192	0.64	Satyanarayana and Johri (1983)
A. terrestris	LSF	Cellulose	45	6.5	72	266	Grajek (1987)
A. fumigatus	SSF	Wheat bran	45	—	72	10 U/g	Kitpreechavanich (1984)
C. thermophile	LSF	Paddy straw	45	7.0	192	0.48	Satyanarayana and Johri (1983)
H. grisea	LSF	Wheat straw	45	—	36	0.55	Thakur et al. (1992)
H. lanuginosa	LSF	Paddy straw	45	7.0	192	0.20	Satyanarayana and Johri (1983)
H. lanuginosa	SSF	Wheat bran	45	—	96	2050 U/g	Kitpreechavanich (1984)
M. albomyces	LSF	Sugarcane bagasse	40	—	96	11	Prabhu and Maheshwari (1999)
M. albomyces	LSF	Xylose	40	6.0	72–96	22.5	Maheshwari and Kamalam (1985)
M. albomyces	SSF	Wheat straw	45	6.0	96	756 U/g	Jain (1995)
M. pusillus	LSF	Cellulose	45	6.5	72	2	Grajek (1987)
S. thermophile	LSF	Rice husk	45	—	168	2.80	Mukherjee et al. (1992)
S. thermophile	LSF	Paddy straw	45	7.0	192	0.79	Satyanarayana and Johri (1983)
T. aurantiacus	LSF	Xylan	50	4.5	288	37.4	Khandke et al. (1989a)
T. aurantiacus	SSF	Sugarbeet	45	6.5	144	9.67	Grajek (1987)
T. lanuginosus	LSF	Birchwood xylan	45/50	6.5	120/144	1613	Puchart et al. (1999)
T. lanuginosus	LSF	Corn cob	45/50	6.5	120/14	2840	Puchart et al. (1999)
T. terrestris	LSF	Solkafloc	48	4.0	30	18.8	Merchant et al. (1988)
T. thermophile	LSF	Paddy straw	45	7.0	192	0.31	Satyanarayana and Johri (1983)
T. thermophila	LSF	Cellulose	45	72	6.5	87	Grajek (1987)

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C. thermophile > *T. aurantiacus* > *Torula thermophila* > *H. lanuginosa* (Satyanarayana and Johri 1983).

Xylanases of thermophilic molds are produced under submerged (SmF) or liquid state (LSF) and solid state fermentation (SSF) conditions (Table 1). Grajek (1987) reported greater xylanase production by *Humicola lanuginosa*, and *Sporotrichum thermophile* in SmF while *T. aurantiacus* produced higher enzyme titres in SSF. *M. albomyces* IIS-68 produced more xylanase in SSF than SmF (Jain 1995). The carbon sources used for xylanase production (Table 1) include natural agro-residues, purified xylan, sugars, and pure cellulose. On agro-residues, co-induction of cellulase occurs along with xylanase since both have a common secretion mechanism (Nevailanen and Palva 1978); sporadic reports of cellulase-free xylanase also exist.

M. albomyces IIS-68 produced appreciable xylanase without contaminating cellulase on sugarcane bagasse (Maheshwari and Kamalam 1985). *T. lanuginosus* produced cellulase-free xylanase under SmF on maize cobs as carbon source (Gomes et al. 1993). Jain (1995) found holocellulose of rice straw as a good substrate for maximal production of xylanase from *M. albomyces* IIS-68 in SSF. The concurrent production of acetylesterase with xylanase on substrates, devoid of acetylated xylan indicated constitutive nature of the enzyme. Dubey and Johri (1987) reported highest xylanase titre in *Sporotrichum* sp. and *Myceliophthora thermophilum* on wheat straw. However, wheat bran is a universal choice for xylanase production without any other carbon and/nitrogen supplementation (Grajek 1987). Xylanase production is affected by the type of xylan, particularly branching, type, and frequency of side groups, and the degree of polymerization of xylan molecule (Jain 1995). Generally, pure xylan is able to support high levels of xylanase (Gruninger and Feichter 1986) however supplementation is required in others (Wong and Saddler 1993).

In a comprehensive study of 22 carbon sources, xylanase production in *T. aurantiacus*, declined in the order, larchwood xylan > alkali treated blotting paper > blotting paper > corn cob > alkali treated bagasse > bagasse; these substrates also induced low level of cellulases (Khandke et al. 1989a). Satyanarayana and Johri (1983) however observed greater production of xylanase on LC (lignocellulosic complex) of paddy straw than on pure xylan and xylose. The order followed by RT9 and MH4 strains of *T. lanuginosus* was, wheat bran < corn cob < xylan (Hoq and Deckwer 1995).

Cellulose, may or may not support xylanase synthesis. Ganju et al. (1989) reported xylanase production in *C. thermophile* var. *coprophile* on microcrystalline cellulose (Sigma cell Type 20). Low xylanase activity on CM-cellulose and Sigma cell type 20 was observed in *T. aurantiacus* (Khandke et al. 1989a). Avicel and loosely woven propylene matrix were reported to support xylanase production (Maijala et al. 1995; Sudgen and Bhat 1994). Xylanase production in *S. thermophile* was quantitatively as well as qualitatively better on wheat straw than on avicel (Sudgen and Bhat 1994).

The improved synthesis of xylanase is affected by the type and concentration of nitrogen source (Hoq and Deckwer 1995). In general, yeast-extract supports maximum xylanase production. Puchart et al. (1999) observed greater production of xylanase and β-xylosidase on corn cob + yeast-extract than on xylan + L-asparagine. *A. fumigatus* NRRL A-27560 produced xylanase along with cellulase on rice straw and spent brewer's grain as sole carbon and nitrogen sources (Coronel et al. 1991). Maheshwari and Kamalam (1985) observed maximum xylanase production in *Melanocarpus albomyces* on 1.5% (w/v) xylose and 0.2% (w/v) urea. The optimized medium for maximum xylanase yield in *T. lanuginosus* contained corn cob (1.35% w/v) and peptone (0.85% w/v) as carbon and nitrogen source (Chadha et al. 1999). The production of xylanase by *T. aurantiacus* IMI 216529 in SSF was enhanced by carbon and nitrogen source, type of inoculum, moisture content, and the particle size; best substrates were wheat straw (particle size < 0.5 mm) > arundo cane > beet pulp and best nitrogen source, ammonium sulfate (Kalogeris et al. 1998).

The temperature maxima for enzyme production in thermophilic molds varies in the range, 40–50°C with 45°C as optimum; pH 6.0–7.0 with 6.5 as optimum (Table 1). An essential prerequisite for good xylanase production by *A. fumigatus* on birchwood xylan was, decrease in pH to less than 3.0; proteolytic enzymes degraded xylanase at higher pH (Bailey and Viikari 1993). In *T. aurantiacus*, an initial pH of 4.5 and temperature at 50°C enhanced xylanase yield (Khandke et al. 1989a). The growth period for enzyme productivity however, generally varies between 2–8 days with maximum after 4 days (Table 1). *T. aurantiacus* produced maximum xylanase on xylan after 12 days (Khandke et al. 1989a).

Besides the above, inoculum size, age of culture, and agitation influence enzyme production. In *T. aurantiacus*, maximum xylanase was observed at 140–180 rpm (Khandke et al. 1989a). A low speed agitation (15 rpm) reduced xylanase production to 19% against the static condition probably due to shear stress sensitivity of the fungal mycelia (Purkarthofer and Steiner 1995). Alams et al. (1994) studied xylanase in *T. lanuginosus* RT 9 and *T. aurantiacus* BJT 190 under SSF. Cellulase-free xylanase was produced on unsupplemented wheat bran by *T. lanuginosus* at 55°C while *T.aurantiacus* produced xylanase along with some cellulase; 0.7% xylan induced xylanase in *T. lanuginosus* leading to 28% increase. Xylanase production in *T. lanuginosus* and *T. aurantiacus* was optimal at 80 and 50% initial moisture content, respectively.

The choice of substrate is critical since besides supporting yield it can also induce the release of a complete complement of xylanolytic enzymes, viz., one xylanase and one β-xylosidase in *T. lanuginosus*, when grown on corn cob (Puchart et al. 1999) besides low levels of α-glucuronidase, acetyl xylan esterase, and AF (Purkarthofer et al. 1993). A growth medium containing oat spelt xylan supported higher level of xylanase, β-xylosidase, and acetyl xylan esterase than the medium containing meadow fescue grass; latter supported

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higher yields of ferulic acid and coumaric acid esterase and *o*-methylglucuronidase in 40 mesophilic and 13 thermophilic organisms (Smith et al. 1991).

Production conditions for AF in *R. pusillus* HHT-I in LSF on L(+)-arabinose were: 40°C, pH 5.5, 150 rpm, and 120 h. Higher yields on L(+)-arabinose, and L(+)-arabitol suggested that synthesis of AF was stimulated by pentoses or by xylan metabolites (Rahman et al. 2001). Gomes et al. (2000) reported good levels of β-xylanase and low levels of β-xylosidase, AF, β-mannase, β-mannosidase, and α-and β-galactosidase in *T. aurantiacus* on a medium containing solka floc and soymeal as carbon and organic nitrogen source.

A direct correlation between mycelial growth and xylanase production has not been observed. In *T. aurantiacus*, pectin and arabinan supported excellent mycelial growth but low enzyme yield; bagasse supported fair mycelial growth and good enzyme yield whereas fructose supported excellent mycelial growth but no enzyme production (Khandke et al. 1989a); xylanase levels were enhanced by Tween-80 whereas $AgNO_3$, $CoNO_3$, $HgCl_2$, $KMnO_4$, $MnCl_2$ and SDS exerted moderate to strong inhibitory effect on enzyme synthesis and activity while $CuSO_4$, EDTA, SnCl, and $ZnCl_2$ exhibited a strong inhibitory effect on activity only (Gomes et al. 1994).

Besides the optimization of culture conditions, enzyme activity can be modified either by structural alterations induced by immobilization or strain improvement through U.V. mutagenesis. Jethro et al. (1993) improved xylanase activity in *M. albomyces* IIS-68 by binding the crude and purified Xyl I and XyII to epoxy carrier. The relative increase in activity of immobilized crude enzyme was 3-fold higher at pH 6.0 and 45°C and 1-fold higher at 40°C. A mutant RB524 of *T. lanuginosus* produced by UV mutagenesis secreted 2.5-fold higher yield than the parent strain (Chadha et al. 1999). Mukherjee et al. (1992) reported three-fold higher xylanase in arg^- and 2-fold higher in ala^- auxotrophic mutants than the wild type in *S. thermophile* on sugarcane baggase. However, Jethro et al. (1993) were unsuccessful in enhancement of enzyme production in *M. albomyces* through protoplasting and subsequent UV mutagenization.

3 REGULATION

Xylanolytic enzyme system is inducible by xylan (Gomes et al. 1994; Maheshwari and Kamalam 1985; Purkarthofer and Steiner 1995) and related hydrolytic products while the synthesis is catabolically repressed by well-metabolized sugars such as glucose and xylose (Gomes et al. 1994; Maheshwari and Kamalam 1985; Purkarthofer and Steiner 1995). Xylan is the most effective substrate-cum-inducer for xylanase biosynthesis (Bastawde 1992; Biely 1985; Dekker and Richards 1976). In its native state, xylan forms a part of insoluble complex comprising mainly of cellulose, pectin, and mannan. The heavily substituted xylan is of very high molecular weight and therefore it is unable to enter the microbial cell. Available data suggest that it is operated through an inducer–receptor complex following a kind of allosteric control. Beechwood xylan, birchwood glucuronoxylan, and methyl-β-D-xylopyranoside (MXP) induced xylanase in *T. aurantiacus*. The most powerful nonmetabolizable inducer, MXP, induced highest xylanase activity among the 14 carbohydrates tested. A low level of constitutive enzyme was produced on all compounds even in the absence of added carbon source to basal medium (Gomes et al. 1994). Xylanases in *S. thermophile* and *A. alabamensis* were induced by xylan, xylose, or a hemicellulose (Satyanarayana and Johri 1983). However, xylose induced very low level of enzyme in *T. aurantiacus* suggesting that this pentose sugar could act as an inducer or repressor depending on the organism (Khandke et al. 1989a). Xylanase in *M. albomyces* IIS68 was inducible only in presence of xylan/xylose; cellulose induced very low levels which is in contrast to the results obtained with some other fungi (Maheshwari and Kamalam 1985). In most cases, natural LCs are better inducers than purified xylan (Puchart et al. 1999; Satyanarayana and Johri 1983); a combination of the two is usually the best (Maheshwari and Kamalam 1985).

Besides xylan, the slowly metabolizable sugars such as acetyl ester of xylobiose and others can serve as gratuitous inducers of xylanase in *T. aurantiacus* (Gomes et al. 1994). However, in *M. albomyces*, methyl-β-D-xyloside and *p*-nitrophenyl-β-D-xyloside were ineffective as inducers as were cellobiose and CMC (Maheshwari and Kamalam 1985). Acetylesterase was however induced by acetylated glucose in *T. lanuginosus* (Puchart et al. 1999). The secretion of xylanase is limited by accumulation of high concentrations of hydrolysis product. The repression by glucose has been reported in *T. aurantiacus* however no derepression was observed with exogenous cAMP supply (Gomes et al. 1994); chitin, raffinose, fructose, lactose, cellobiose, glucose, salicin, and succinate also repressed the enzyme synthesis (Khandke et al. 1989a). The co-production of xylanases and cellulases through a common secretion mechanism (Nevailanen and Palva 1978) and cross-specificity makes the regulatory mechanisms difficult to study; differential regulation has however been reported (Hrmova et al. 1984).

4 PURIFICATION OF XYLANASES

Xylanases from thermophilic molds differ in their physicochemical properties due to which there is no uniform method of enzyme purification. The first step is usually either acetone (Anand et al. 1990) or ammonium sulphate precipitation (Anand et al. 1990; Ganju et al. 1989; Hayashida et al. 1988; Khandke et al. 1989a,c; Matsuo and Yasui 1988; Prabhu and Maheshwari 1999; Rahman et al. 2001) followed by desalting on Sephadex G-25 (Anand et al. 1990; Ganju et al. 1989; Khandke et al. 1989c) or dialysis against a buffer (Matsuo and Yasui 1988). Xylanase from *H. lanuginosa* was purified four-fold by ion-exchange chromatography on DEAE-Sephadex A-50 followed by QAE-Sephadex A-50 and gel-filtration on Bio-Gel-P-30 (Anand et al. 1990). Xylanase from *T. aurantiacus* was purified by DEAE-Sephadex A-50 and Ultrogel AcA-54 column chromatography (Khandke et al.

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Table 2 Properties of purified β-1,4-endo-xylanases from thermophilic molds

Organisms	Multiplicity of xylanase	Optimum		Stability		K_m	V_{max}(μM/min/mg)	pI	M.W. (KDa)	Inhibitors	Hydrolysis products	Cellulase activity	Reference
		PH	Temp. (°C)	pH	Temp. (°C)								
C. thermophile var. *coprophilile*	Xyl I	5.4–6.0	70		50	0.55 mg/ml	58.8	8.6	26	$HgCl_2$ and $KMnO_4$	X_2, X_3 and traces of X_n		Ganju et al. (1989)
	Xyl II	4.8–6.4	60		50	0.1 mg/ml	5.26		7		Mainly X_2		
H. grisea var. *thermoidea*	Xyl I	6.0	60		60–100	2×10^{-3} M	18.18		98		X_2, X_3, X_4 and traces of X_1	+	Thakur et al. (1992)
	Xyl II	7.0	60		60–100	3.3×10^{-3} M	20		13		X_2, X_3, X_4 and traces of X_1	−	
H. lanuginosa		6.0	65	6.0l–9.0	50	0.91 mg/ml	833		22.5			−	Anand et al. (1990)
M. pulchella var. *sulfurea*		6.0–6.5	70		50						X_1, X_2, X_3		Matsuo and Yasui (1988)
M. albomyces von *Arx.* IIS-68	Xyl IA	6.6	65	5.0l–10.0		0.3 mg/L	311		38		X_3, X_2	−	Prabhu and Maheshwari (1999)
	Xyl III A	5.6	65	5.0l–10.0		1.69 mg/L	500		24		X_n, X_3, X_2	−	
T. byssochlamydoides YH-50	Xa	5.5	75	3.0–9.0	Up to 70°C				76		Xyl, Arab, Gluc, X_2,X_3, Xn	+	
	XbI	4.5	70	3.5–8.5					54		—	+	Hayashida et al. (1988)
	XbII	5.0	70	3.0–9.0					45		X_2, X_1	+	
T. emersonii	Xyl II							5.3	131				
	Xyl III							4.2	54.2				Tuohy et al. (1993)
T. aurantiacus	Xyl I	4.0–4.5	70–75										Kalogeris et al. (1998)
	Xyl II	4.0–4.5	70–75										

X_1—Xylose, X_2—Xylobiose, X_3—Xylotriose, X_n—Xylooligosaccharides (*n* is usually greater than 5), Arab—Arabinose, Gluc—Glucose, + Present, − Absent.

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1989a). Thakur et al. (1992) fractionated the multiple xylanase of *Humicola grisea* var. *thermoidea* on Sephadex G-100 into three peaks, only first and the last had xylanase acivity that was further purified by subjecting it to SDS-PAGE. Xylanase from *S. thermophile* (Mukherjee et al. 1992) was fractionated into three active fractions—xyl a, xyl b, and xyl by consecutive chromatographying on Sephadex A-50 and Sephadex G-100 columns.

Use of various chromatographic techniques has led to firm establishment of multiple forms of xylanases. Hayashida et al. (1988) purified multiple xylanases of *Talaromyces byssochlamydoides* through, Sephadex G-50, DEAE-Sephadex A-50, and Sephadex G-100. Xylanase from *M. albomyces* von. Arx strain IIS68 was purified (Prabhu and Maheshwari 1999) on DEAE-Sephadex A-50 and Ultrogel AcA-54; four major peaks were obtained, of which three were active on larchwood xylan (Xyl I, Xyl II, and Xyl III). The multiplicity of xylanase usually poses problem in purification and requires elaborate procedures. The small amount of purified protein results in paucity of information on the enzymes from any single organism. The α-D glucuronidase from *T. aurantiacus* was purified 394-fold by consecutive fractionation on DEAE-Sephadex A-50 and Ultrogel AcA-54 columns with a yield of about 9.8% (Khandke et al. 1989c). Ganju et al. (1989) purified multiple xylanases (Xyl I and Xyl II) of *C. thermophile* var. *coprophile* on DEAE-Sephadex A-50 and Sephadex G-75 columns. Matsuo and Yasui (1988) purified xylanase of *Malbranchea pulchella* var. *sulfurea* by DEAE-Cellulose, XM (100) Amicon and UM10 ultrafiltration membrane and CM-Sephadex column achieving a 161-fold purification. Rahman et al. (2001) achieved four-fold purification of AF from *R. pusillus* HHT-1 by chromatography on Superdex 200 PG (Pharmacia), CM-Toyopearl650 M (Tosoh Co., Tokyo), and Poros QE/M.

5 PROPERTIES

The physico-chemical characterization of xylanases is essential for understanding the functionality of each component under different biophysical conditions. The enzymes from geographically distinct fungal isolates differ not only in enzyme productivity but also in structural and bio-chemical properties (Kitpreechavanich et al. 1984; Cesar and Mrsa 1996). In general, xylanases are single-chain glycoproteins of molecular weight, 6–80 KDa, active in temperature range 55–65°C, at pH 4.5–6.5 (Prabhu and Maheshwari 1999). Xylanases from thermophilic molds are also glycoproteins (Table 2) with considerable amount of carbohydrate, which is either covalently linked to the protein or present as dissociable complex (Wong et al. 1988). The glycolipid fraction appears to play a role in stabilizing the protein structure and in enhancement of thermostability (Woodward 1984). The multiplicity may also be a consequence of this feature (Wong et al. 1988).

Thirteen endoxylanases purified from *T. emersonii* were glycoprotein; carbohydrate moiety provided thermostability (Tuohy et al. 1994). While *H. lanuginosa* xylanase contained 1.18% carbohydrate (Anand et al. 1990), three xylanases from *T. byssochlamydoides* YH-50 (Xa XbI, and XbII) contained 36.6, 31.5, and 14.2% carbohydrate which were rich in glucose with lesser amounts of mannose and fucose (Hayashida et al. 1988). *M. albomyces* IIS-68 xylanase contained 7% carbohydrate (Prabhu and Maheshwari 1999). In *A. fumigatus*, removal of 37% carbohydrate by periodate oxidation had virtually no effect on specific activity but only 3% of maximum activity remained after pretreatment at 65°C for 40 min compared to 53% of the untreated native enzyme (Flannigan and Sellars 1978).

5.1 Molecular Weight

The molecular weight of xylanases of thermophilic molds varies between 7–131 KDa (Table 2). The smallest known fungal xylanase is from *C. thermophile* var. *coprophile* of 7 KDa (Ganju et al. 1989); the largest is from *A. fumigatus*, a 209 KDa protein composed of two subunits (Ximenes et al. 1997). A dimeric β-xylosidase of 181 KDa was reported from *T. emersonii* (Tuohy et al. 1994). α-D-Glucuronidase of *T. aurantiacus* is 118 KDa (Khandke et al. 1989c). Anand et al. (1990) reported a 29 KDa xylanase from *H. lanuginosa*. Silva et al. (1994) reported one xylanase isozyme from *A. fumigatus* of molecular weight 109 KDa, composed of two equal subunits. Microbial endoxylanases show a strong relationship between molecular weight and pI. Wong et al. (1988) grouped the endoxylanases into two categories, (a) low-MW/basic (below 30 KDa), and (b) high-MW/acidic (above 30 KDa). AF of *R. pusillus* HHT-1 is of the size 88 KDa with a pI 4.2 (Rahman et al. 2001). However, exceptions to this general rule have been reported (Wong et al. 1988).

5.2 Thermal Stability

The most characteristic feature that makes xylanases of thermophilic molds stand apart is their temperature optima (T_{opt}) and thermostability. These two features have attracted increased attention owing to their biotechnological potential. The optimum temperature for activity of xylanases is in the range, 50–70°C; thermostability reaches upto 100°C (Table 2). Thermostable xylanases of *Humicola* sp. had a high of T_{opt} 75°C (Silva et al. 1994), while those from *S. thermophile* exhibited lower T_{opt} of 50°C (Mukherjee et al. 1992). Xylanases from *H. grisea* var. *thermoidea* were thermostable between 60–100°C (Thakur et al. 1992). The two xylanases (Xyl I A and Xyl III A) from *M. albomyces* von. Arx IIS-68 were maximally active at 65°C but less stable at this temperature than at 50°C. Xyl I A exhibited greater thermostability than Xyl III A; it retained 60–80% of its activity at 50°C even after 72 h of incubation while Xyl III A lost 40–60% activity in 2 h at 50°C with no further loss upto 24 h (Prabhu and Maheshwari 1999). In *Chaetomium*

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thermophilum var. *coprophile*, both Xyl I and Xyl II lost 50% activity at 60°C but they were stable at 50°C for 24 h (Ganju et al. 1989). Three xylanases of *T. byssochlamydoides* were stable up to 70°C and retained 65, 44, and 29% of original activity after heating at 95°C for 5 min (Hayashida et al. 1988). However, xylanase of *M. pulchella* was completely inactivated at 70°C within 5 min (Matsuo and Yasui 1988). Tan et al. (1987) reported an extremely thermostable xylanase from *T. aurantiacus* with T_{opt} of 50°C; it retained total activity after 24 h at 70°C or 97 h at 60°C; its half-life at 80°C was about 60 min. Endoxylanases from *T. emersonii* were maximally active at 73–80°C (Tuohy et al. 1994). Xylanase of *H. lanuginosa* was stable between, 30–60°C for 60 min, with 90% loss at 70°C (Anand et al. 1990). The crude enzyme of *T. lanuginosus* was optimally active at 70–75°C and retained about 90% activity after 41 h preincubation at 55°C; even after 188 h, there was only 20–30% loss in activity (Gomes et al. 1993). α-D-glucuronidase from *T. aurantiacus*, with a T_{opt} of 60°C, retained 42% activity at 70°C after 6 h. It was rapidly inactivated at 70°C, retaining only 24% activity after 60 min. In contrast, incubated at 70°C for 60 min in the presence of substrate, the enzyme retained 87% of the activity obtained at 65°C and 156% of that obtained at 50°C (Khandke et al. 1989c). The T_{opt} of AF of *R. pusillus* HHT-1 was 60°C and stability upto 70°C (Rahman et al. 2001).

Thermal stability of crude β-xylanase from nine strains of *T. lanuginosus* was variable. Strain SSBP retained 100% activity for over 48 h at 65°C whereas seven ATCC strains and strain DSM 5826 retained 100% activity up to 60°C. The xylanase produced on corn cob was most thermostable and showed 6% increase in activity from 70 to 80°C. $T_{1/2}$ value for all strains at 70°C and pH 6.5 varied between 63 min (ATCC 28083) to 340 min (SSBP). Strain SSBP therefore appeared suitable for industrial applications (Suren et al. 2000).

5.3 pH Stability

The optimum pH for xylanases of thermophilic molds varies between 4.0–7.0 (commonly around 5.0) while pH stability lies in the range, 3.0–10.0 (Table 2). Optimum pH for xylanase from *T. aurantiacus* was 5.2 (Khandke et al. 1989a). Xylanases from *T. lanuginosus* and *T. aurantiacus* were optimally active at pH 6.0 and 5.0, respectively, (Alams et al. 1994). Two xylanases with pH_{opt} 5.0 and 5.6 were reported from *Humicola* sp. (Silva et al. 1994). Endo-β-xylanase of *T. lanuginosus* was stable in pH range, 6.0–9.0 (Lischnig et al. 1993). The crude xylanase of *T. lanuginosus* SSBP exhibited stability in pH range 5.0–10.0 (Suren et al. 2000). *H. lanuginosa* xylanase was stable over pH range, 6.0–9.0 for 24 h at room temperature (Anand et al. 1990). β-Xylosidase of *A. fumigatus* exhibited maximum activity between pH 4.5 and 5.0 (Kitpreechavanich 1984). All endoxylanases of *T. emersonii* were optimally active in pH range 4-4–7.0 (Tuohy et al. 1993). A pH of 6.5 was optimum for xylanase of *T. thermophila* and *Mucor pusillus* while pH 4.0 was most suitable for maximal activity in *T. aurantiacus* (Grajek 1987).

α-D-Glucuronidase from *T. aurantiacus* had pH_{opt} of 4.5 and pH stablity in the range, 3.0-7.0 (Khandke et al. 1989c). The crude enzyme of *T. lanuginosus* was optimally active at pH 6.5 and stable from 6.5–9.0 (Gomes et al. 1993b). The AF of *R. pusillus* HHT-1 was active at pH 4.0 and stable from 7.0–10.0 (Rahman et al. 2001). A pH of 6.5 was optimum for several fungal xylanases (Grajek 1987).

5.4 Effect of Metals, Activators, and Inhibitors

$HgCl_2$ and $KMnO_4$ were inhibitory for *T. byssochlamydoides* xylanase (Yoshioka et al. 1981). However, xylanase from *T. aurantiacus* was drastically affected by $CuSO_4$, $ZnSO_4$, $CaCl_2$, $CoCl_2$, $BaCl_2$, $MgSO_4$, and EDTA at 2 mM concentration (Table 2); at this concentration, $HgCl_2$ abolished complete activity while lead caused only 25% reduction (Tan et al. 1987). Li^{2+}, Na^{+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Ba^{2+} inhibited AF activity in *R. pusillus* HHT-1 by 20% wheras 30% loss in activity occured by Ti^{2+}, Sr^{2+}, 60% by EDTA, and 90% by Cu^{2+} and Hg^{2+}. This enzyme was sensitive to sulfhydryl inhibitors confirming existence of essential thiol groups in the enzyme (Rahman et al. 2001).

The storage of enzyme is an important characteristic for commercial exploitation. Xylanase from *T. lanuginosus* and *Thermoascus aurantiacus* was active after prolonged storage (Alams et al. 1994); no loss was observed after 1-month storage at 4°C whereas about 90% activity remained after 10 d at 55°C. The first report of loss of specific activity on storage for a purified xylanase in dry state at −20°C for longer than 2 months was made by Anand et al. (1990). The loss of specific activity on account of some structural changes in the protein was associated with the appearance of heterogeneity in the protein samples, as indicated by NATIVE-PAGE and the sedimentation pattern; charge and size of the protein under prolonged storage, (up to 2 months) were the likely reasons for loss in enzyme activity. The authors have concluded the inactive protein to be a polymer of native protein on account of: Gel filtration behavior, ultracentrifugation, and SDS-PAGE profile. The phenomenon of inactivation therefore requires further experimentation.

5.5 Substrate Specificity and Mode of Action

Xylanases are known to show wide substrate specificity besides cross-specificity towards cellulosic substrates since both the enzymes compete for the same site. Xylanase from *H. lanuginosa* was quite specific for xylan with no action on crystalline cellulose, CMC, starch, larchwood xylan, *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-xyloside, and polygalacturonic acid (Anand et al. 1990). Endoxylanases of *T. emersonii* were capable of hydrolyzing various xylans with different specificities; some components of the enzyme

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complex showed transferase and hydrolase activity (Tuohy et al. 1994).

The mode of action of only a few microbial xylanases has been studied either by kinetic or by products analysis. K_m of xylanases of thermophilic molds ranges between 0.27 and 14.0 mg ml^{-1} of substrate and V_{max} between, 5.0 and 850.0 μ mol min^{-1}mg^{-1} (Table 2). The two xylanases from *S. thermophile* (Xyl a and Xyl b) had a K_m of 15.4 and 10 mg ml^{-1} and V_{max} of 0.040 and 0.067 U, respectively. These were apparent V_{max} and K_m since the products of xylan hydrolysis themselves become substrate for further hydrolysis (Mukherjee et al. 1992); AF of *R. pusillus* had a K_m of 0.59 M and V_{max} of 387 μmol min^{-1}mg^{-1} (Rahman et al. 2001). The K_m and V_{max} of α-D-glucuronidase from *Thermoascus aurantiacus* was, 0.145 mM and 2.5 μmol min^{-1} mg^{-1} (Khandke et al. 1989c).

The xylanase family in *Chaetomium thermophle* var. *coprophile* consists of members with varying hydrolytic pattern (Ganju et al. 1989). The reaction products of Xyl I consisted mainly of xylobiose, xylotriose, and minor amount of higher oligosaccharides indicating that it cleaves xylan molecule randomly (endo-). Xyl lI released mainly xylobiose as reaction product, suggesting that it degrades xylan by removing one xylobiose molecule at a time (exo-). *A. fumigatus* Fresenius and *H. grisea* var. *thermoidea* xylanases degrade xylan by an endo-type action forming intermediate molecular weight substituted and unsubstituted products alongwith traces of xylose. Xyl I of *A. fumigatus* showed transglycosylation reaction since it released xylobiose, xylotriose, and smaller amounts of higher xylooligomers but no xylose from xylotetraose. The hydrolysis of kraft pulp by Xyl II of *H. lanuginosa* released xylobiose and xylohexose while action on sulphite pulp resulted in a mixture of xylobiose, xylopentaose, and higher xylooligomers. The two endo xylanases (Xyl I and Xyl II) of *H. grisea* var. *thermoidea* released xylobiose, xylotriose, xylotetraose, and traces of xylose (e Silva et al. 2000).

The xylanase of *M. pulchella* hydrolysed xylan to approximately 51% and showed transglycosylation reaction in degradation of both xylo-tetraose and xylose−pentaose (Matsuo and Yasui 1988). The three xylanases (Xa, XbI, and XbII) of *T. byssachlamydoides* YH-50 hydrolyzed xylan to 38, 70, and 75% in 5 d while a combined action of the three hydrolysed it to 90% in 5 d with weak activity toward CMC, Avicel, starch but no activity towards xylobiose, cellobiose, or maltose (Hayashida et al. 1988). Xylanases of *M. albomyces* IIS 68 differed in the rate of hydrolysis of xylans. Maximal saccharification of larchwood, oatspelt xylan, and sugarcane baggase by Xyl IA was 30, 38, and 29% while that by xyl III A, 26, 27, and 18%, respectively, and both were endo-acting (Prabhu and Maheshwari 1999). Khandke et al. (1989b) made a detailed study of the degradation of birchwood xylan by *Thermoascus aurantiacus*; percentage hydrolysis was 50 with purified xylanase and 93 with crude protein. The partial hydrolysis of larchwood xylan produced neutral and acidic xylooligosaccharides, which confirmed that the enzyme was an endoxylanase; no further hydrolysis of the tetrasaccharide showed that it did not prefer to act at the bond involving xylose residue to which acidic sugar was attached in branch and adjacent xylose. Xylanase rapidly hydrolyzed xylopentaose into xylotriose and xylobiose indicating that the preferred site of action was xylosidic bond between the second and third xylose residue starting from the reducing end.

Gilbert et al. (1993) compared the mode of action of xylanases from *Thielavia terrestris* 255 B and *Thermoascus crustaceus* 235 E, both of which belong to two different families. Xylanases from *T. terrestris* achieved faster solubilization of insoluble xylan compared to *T. crustaceus*, which was more effective in producing xylose and short xylooligomers. Their combined hydrolytic potential did not reveal any cooperative action, which could be due to similar role played by these xylanases in substrate cleavage. The xylanolytic enzyme system of *T. emersonii* comprising of one β-xylosidase and two xylanases was active against a variety of substituted xylans hydrolyzing aryl-β-xylosides as well as unsubstituted xylan; the stearic hindrance for endoxylanases by side-chain substituents was observed in case of branched xylans (Tuohy et al. 1993). The α-D-glucuronidase of *T. aurantiacus* hydrolysed 4-*o*-methyl-α-D-GpA into xylotriose, xylobiose, and xylose; it cleaved only α-1,2-D-glycosidic bond but did not attack the β-1,4 linkage in the xylose backbone. This enzyme hydrolysed 4-*o*-methyl-α-D-GpA-Xyl_1 to Xyl_7 at rates comparable to xylan but exhibited lower activity on starch, maltose and *p*-nitrophenyl-α-D-cellobiose, α-cyclodextrin,(*p*-nitrophenyl-β-D)-glucoside, -xyloside, xylo -biose, -triose, -tetraose, and -pentaose (Khandke et al. 1989c).

When tested on various synthetic sugars coupled to *p*-nitrophenol, AF of *R. pusillus* was active only on *p*-nitrophenylarabinofuranoside (pNPA); specificity was restricted to furanose form of L(+)-arabinose rather than pyranose. Higher activities were observed with arabinotriose and sugarbeet arabinan and little with arabinoxylan and arabinogalactan. As an exo-acting enzyme, it liberated L(+)-arabinose as terminal product from pNPA, polyarabinan, and low molecular mass arabino-oligosaccharides such as sugarbeet, arabinan, and arabinotriose. Based on the action and substrate specificity, it was found to belong to *Streptomyces purpurascens* type of AF (Rahman et al. 2001). The formation of end products is however, dependent on the enzyme dosage; lesser enzyme dose liberates xylooligomers with DP upto 7 while higher one results in greater production of xylose, xylobiose, and xylotriose (Archana et al. 1997).

The difference in catalytic properties of xylanases from a microorganism is on account of xylosidic linkages in heteroxylan, which are not equally susceptible to enzymic hydrolysis. The sites of attack on the main chain and relative rates of hydrolysis of linkages close or far from a substituent are goverened by the architecture of the enzyme substrate-binding site. The substrate-binding site is envisaged as an array of subsites on the protein molecule, each binding or accomodating one xylopyranosyl residue of the main chain, with catalytic groups between two such subsites. The rates of

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hydrolysis of glycosidic linkages around a substituent may differ by an order of magnitude, however, only some of these linkages remain readily inaccessible for enzyme cleavage. The differences in the extent of heteroxylan hydrolysis are therefore a consequence of size of the substrate-binding site. Thus, an enzyme with larger substrate binding site requires larger unsubstituted part of the main chain than the enzyme with smaller sites. Therefore, the former will hydrolyze heteroxylans to smaller extent and generate products larger than the latter. Thus, an enzyme with larger binding site shows a much higher increase in affinity for linear xylooligosaccharides than those with smaller substrate binding sites (Biely et al. 1992).

5.6 Multiplicity of Xylanases

Xylan is a heteropolysaccharide in which the xylosidic linkages are not equivalent and equally accessible to xylanolytic enzymes. Furthermore, accessibility of at least some linkages changes during the course of hydrolysis. The complete hydrolysis of xylan, therefore, involves not only a spectrum of enzymes but also production of more than one xylanase (i.e., multiplicity) by a microorganism (Wong et al. 1988). Multiplicity refers to production of more than one type of xylanase by a species that differs in molecular size, stability, substrate-binding site, adsorption, or activity on insoluble substrate and in the number of unsubstituted xylose units required in the substrate–polysaccharide backbone to facilitate hydrolysis (Biely 1985; Coughlan and Hazlewood 1993).

Multiple xylanases have been reported in a number of thermophilic molds (Table 2). Few multiple xylanases have however been well characterized due to difficulty in their purification. The extent of xylanase multiplicity remains to be answered. Five xylanases were reported from *Chaetomium thermophilum* var. *coprophile* (Ganju et al. 1989) and thirteen endoxylanases from *T. emersonii* (Tuohy and Coughlan 1992). The zymogram analysis revealed one major and two minor xylanases in *T. auratiacus* (Khandke et al. 1989a). The nature and relevance of minor xylanases remains elusive since purification procedures favor isolation of major protein. The function is not clearly known but it is assumed that they are involved in subsidiary functions not required in large quantities like hydrolysis of linkages that occur infrequently. They are either not produced in large quantities or are lost from culture filtrate due to degradation or adsorption onto insoluble growth substrate (Wong et al. 1988).

The substrate plays an important role in induction of multiple xylanases. A complete set of xylanolytic enzymes including 13 endoxylanases, 1 β-D-xylosidase, 1 arabinofuranosidase, 3 acetyl esterase alongwith some other enzymes were produced in *T. emersonii* when grown on appropirate substrate (Tuohy and Coughlan 1992). The basis of xylanase multiplicity can be properly assessed only by more comprehensive studies comprising regulation, cross-specificity, and post-translational modifications of the enzyme since each of these factors influences multiplicity (Wong et al. 1988). A majority of multiple fungal xylanases are allozymes i.e., products of different alleles of the same gene. Alternatively, each of the multiple xylanase may be a distinct gene product produced by a fungal species. The same explanation applies to multiplicity of β-D-xylosidase (Wong et al. 1988). Various explanations have been provided to account for xylanase multiplicity, of which, the most relevant ones are (Biely 1985; Coughlan and Hazlewood 1993; Gilbert and Hazlewood 1993; Wong et al. 1988):

- Heterogeneity in xylan structure, which leads to production of endoxylanases of different specificities yielding quite different products often produced by the same microbial strain.
- Different biophysical environments in which a microbial species grows.
- Differential mRNA processing and posttranslational modifications such as glycosylation or proteolysis or both. Many xylanases are glycosylated and some are apparently translated as precursor with peptide signal sequence.

Two xylanases with similar hydrolytic, immunological, and physico-chemical properties must be differentially modified products of the same gene (Wong et al. 1988). The multiplicity could be due to secondary nonspecific changes such as aggregation of enzyme molecule (Gilbert and Hazlewood 1993). It is also possible that some of the xylanases in the culture filtrate are precursors of or degradation products of other xylanases. The multiplicity could also arise from strain impurity or inability of the assay substrate to differentiate between different classes of enzymes.

The relevance of xylanase multiplicity lies in the function of each component of the complex. It has been concurred that if multiple xylanases are separate gene products then the individual xylanases have distinct properties contributing key functions to overall xylanolytic system of the microbial species. These distinct functions are conserved among the xylanolytic systems. The significance of multiple xylanases also lies in the efficient hydrolysis of xylan substrate, which is the central function, and in hydrolysis of other xylose containing polysaccharide as secondary substrates. Since, xylan is an important component in fibre cohesion, the primary function of multiple xylanases is related to destruction of fibre integrity and exposure of other lignocellulosic components to action of hydrolases. Such functions may be related to debranching and/transferase activities in some xylanases rather than only to hydrolysis of xylosidic linkages. In majority of the cases, however, multiple xylanases exhibit synergism in hydrolysis of xylan. Dekker and Richards (1976) proposed that the complexity of xylan requires the action of multiple xylanases with overlapping yet different specificities to effect extensive hydrolysis.

Thakur et al. (1992) have confirmed the multiplicity of xylanases immunologically in *H. grisea* var. *thermoidea*;

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immunodiffusion test resulted in formation of two precipitin bands with crude culture filtrate and a single band with two purified xylanases (Xyl I and Xyl II). The extent of hydrolysis of oatspelt xylan was much greater with a mixture of Xyl I and Xyl II compared to individual preparations suggesting cooperative action. The saccharification of xylan by a combination of the three xylanases of *M. albomyces* in 20 min was equal to the airthmetic sum of the saccharification by the individual enzymes showing absence of synergism in their action. The functional significance of the multiple forms where synergism is absent, lies in their ability to function under different conditions on account of their different physico-chemical properties (Prabhu and Maheshwari 1999).

6 MOLECULAR STRUCTURE

Endo-xylanases are classified into two distict families, F/10 and G/11, a component of 60 distinct families in which glycosyl hydrolases have now been classified based on amino acid sequence identities in their catalytic domains and hydrophobic cluster analysis (Davies and Henrissat 1995; Henrissat 1991; Henrissat and Davies 1997). They possess three to five subsites for binding the xylopyranose rings close to the catalytic site. Family F xylanases are of high molecular weight, low pI, low thermostability, more complex, exhibit low substrate specificity towards β-xylan, and produce smaller oligosaccharides. Family G xylanases are of low molecular weight, high pI, high thermostability, exhibit low substrate specificity but produce higher oligosaccharides; they are more industrially relevant. The multiple xylanases produced by an organism may belong to either different or same families.

The structure of F/10 family xylanases is $(\alpha/\beta)_8$ TIM-barrel type while that of family G/11is predominately β-sheet (Natesh et al. 1991). The catalytic domain of Family F xylanases is cylindrical a/b barrel resembling a salad bowl with the catalytic site at the narrow end i.e., near the C-terminus of the β barrel. The catalytic domain in family G consists principally of β-pleated sheets formed into a layered trough that surrounds the catalytic site; protruding into trough, and located towards one side of the protein is a long loop which terminates in an isoleucine. The two families of xylanases, inspite of their structural differences, function in a similar manner by double displacement mechanism of enzymatic hydrolysis (Henrissat and Davies 1997). The mode of action of these enzymes is governed by of their 3-d structure rather than the globular fold (Davies and Henrissat 1995).

To date, crystal structure of xylanases of two thermophilic molds has been reported (Kumar et al. 2000; Natesh et al. 1991). Viswamitra et al. (1993) crystallized an endoxylanase of molecular weight 29.77 KDa of *Thermoascus aurantiacus*, as monoclinic pinacoids in presence of ammonium sulphate buffered at pH 6.5 and also with neutral polyethylene glycol 6000. Crystals belonged to the space group $P2_1$ and had cell dimensions, $a = 41.2$ Å, $b = 67.76$ Å, $c = 51.8$ Å, $\beta = 113.2°$. This xylanase has been reported to function autonomously without cellulose binding domain in its molecular structure. It is the first reported thermostable F/10 xylanase with a high degree of activity and specificity and therefore could be an industrially important structure (Natesh et al. 1991). The structure of this xylanase was also determined by molecular replacement method using *Streptomyces lividans* xylanase module. The aminoacid sequence determined from 1.8A° resolution electron density map aided by multiple sequence alignment with related xylanases solved the errors in the primary sequence reported earlier (Srinivasa et al. 1990; Srinivasa et al. 1991). It has 301 aminoacid residues and 261 water molecules. The single polypeptide chain assumes the typical $(\alpha/\beta)_8$ TIM-barrelfold comprising 8 major parallel β-strands, which are nearly side-by-side in the form of a cylinder at the centre surrounded by 8 major helices. The PROCHECK analysis of the secondary structure revealed six extra short helices dispersed along the polypeptide chain. The active site consists of two glutamate residues, Glu 131(acid/base) at C-terminus of β4 and Glu 237 positioned towards the C-terminus of β7. It follows the typical double displacement mechanism of substrate hydrolysis as the average distance between glutamate residues, Glu 131 and Glu 237 side chain carboxylate oxygen atoms is 6.55 Å i.e., close to 5.5 Å which is expected for retaining this mechanism. The three conserved histidine residues, His 83, His 209, and His 88 are involved in hydrogen-bond network in the vicinity of the active site glutamate residues, which appear to be responsible for maintaining the ionization state of the two catalytic Glu residues. The salt bridge Arg 124−Glu 232, which is almost buried, bridges the β strands β_4 and β_7 and could contribute towards thermostability and activity of the enzyme. It is the first reported F/10 xylanase, which has a proline residue in the middle of α-helix α6; this could contribute towards better packing leading to a more stable structure and thus higher thermostability. The short helices engineered between secondary structural elements known as thermohelices are also reported to contribute towards thermostability by shortening the connecting loops and making them more regular. The smaller the thermal motions, as represented by smaller B values (9.9 and 12.8A$^{\circ 2}$, for the main- and side-chains respectively) together with the observed nature of inter-molecular interactions suggests that the molecule is less susceptible to attack as temperature increases.

The crystal structure of endoxylanase of *Paecilomyces varioti* Bainier, a thermophilic member of family G/11 (stable up to 75°C), conforms to the space group P $2_12_12_1$ with $a = 38.76$ Å, $b = 54.06$ Å, and $c = 90.06$ Å. The structure of this xylanase was resolved by molecular replacement technique using polyalanine coordinates of *T. lanuginosus* xylanase. It has 194 amino acid residues and 128 water molecules with a crystallographic R-factor of 19.07% and a free R-factor of 21.94%. It has two-curved β-sheets, forming cylidrical active-site cleft, and a lone α-helix, as present in other family-11 xylanases. A quantitative comparison of structure and sequence of xylanase of this fungus with those

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from mesophiles and thermophiles suggests that the "hinge" region is made more compact in thermophiles on account of addition of a disulphide bridge between Cys110 and Cys554 and a N–H...O hydrogen bond between Trp159 near the extremity of the lone α-helix and Trp138 on β-strand β8. This study explicitly brings out the presence of the C–H...O and the C–H...π type interactions in xylanases (Kumar et al. 2000).

The amino acid composition of a xylanase from *C. thermophilum* var. *coprophile* has revealed that this protein contains higher amount of glycine, threonine, aspartic acid–asparagine, glutamic acid–glutamine, and lower amount of sulphur containing amino acids (Ganju et al. 1989). *H. lanuginosa* xylanase has fair abundance of aspartic and glutamic acids as well as glycine; low cysteine content and total absence of methionine. It has arginine as N-terminal amino acid (Anand et al. 1990). *T. byssachlamydoides* xylanase exhibits abundance of aspartic and glutamic acid residues (Hayashida et al. 1988). The N terminus sequencing of AF of *R. pusillus* HHT-1 revealed the first 22 amino acids as: L-N-L-T-I-Y-N-D-L-P-G-G-S-T-T-P-L-Q-R-G-I-M. This sequence showed no homology with that of any known AF of microbial origin after BLAST analysis (Rahman et al. 2001).

7 APPLICATION POTENTIAL

The potential application of xylanases is in industries associated with pulp and paper, food and feed, textile, and bioconversion (Kuhad et al. 1997). Among these sectors, xylanases are most sought after enzymes in pulp and paper industry. Residual lignin (3–5%) is the major drawback of the kraft process of paper manufacture. It imparts an undesirable brown color to the pulp, which requires a subsequent bleaching step. Bleaching with Cl_2/ClO_2 results in brown colored derivatives of lignin which comprises of dioxins and related compounds that are toxic, carcinogenic, and recalcitrant and therefore pose serious environmental hazards (Viikari et al. 1986; 1994; Srinivasan and Rele 1995). The alternative bleaching agents have their own limitations, such as O_2 (widely used but requires considerable investment); H_2O_2 (expensive chemical to use industrially) and O_3 (bleaching performance is good but cellulose degradation results in loss of fibre strength).

The quest for economically viable and eco-friendly alternatives infused interest in unraveling the potentiality of cellulase-free xylanases in paper and pulp industry to produce the rayon grade paper pulp and superior quality dissolving pulp. Xylanases can be used at different stages of pulping process but the most important application is in pre-bleaching of Kraft pulp where it reduces the amount of ClO_2 compounds used in the bleaching step (Srinivasan and Rele 1995). Pre-treatment with xylanases causes substantial reduction in residual lignin and thus allows saving in bleach chemicals, reduced AOX (adsorbable organic halide) and dioxins. Xylanase enhances pulp brightness and causes reduction in Kappa number in the final paper product. It also increases pulp fibrillation and water retention, reduction of beating time in virgin pulps, restoration of bonding and increased freeness in recycled fibres and selective removal of xylan from the dissolving pulp (Srinivasan and Rele 1995).

The emphasis on Cl_2-free technology on global scale has resulted in rapid availability of commercial preparations such as Pulpzyme HA (NovoNordisk A/S), Irgazyme 10 and Irgazyme 40 (Genencor, International), Cartazyme HS and HT (Sandoz), Ecopulp (Alko Ltd.) and VAI xylanase (Voest-Alpine). Among these, VAI xylanase is the first commercial preparation from thermophilic mold *T. lanuginosus* which induces a 2.5 brightness point increase or a 31% decrease in chlorine consumption (Bajpai et al. 1994). Several strains of this thermophile produce cellulase-free xylanases active and stable at high temperature and neutral pH values (Alams et al. 1994; Hoq and Deckwer 1995; Suren et al. 2000). A crude xylanase preparation of *T. lanuginosus* DSM 5827 was able to solubilize 27% xylan present in original bleached dissolving pulp (Giibitz et al. 1997) while strain MED 2D decreased the Kappa number of the eucalyptus pulp by 11% (Haarhoff et al. 1999). Silva et al. (1994) reported that the treatment of bleached Kraft eucalyptus pulp by xylanase of *Humicola* (Xyl I, t_{opt} 75°C, pH 5–5.6) and its crude enzyme preparation enhanced pulp brightness and increased pulp viscosity. Christov and Prior (1997) tested crude xylanase preparation from *T. lanuginosus* to remove hemicellulose and lignin from sulfite pulps in conjunction with H_2O_2 in one-stage and multi-stage bleaching of dissolving pulps; some biobleaching effect was observed in one stage H_2O_2 treatment but no improvement in brightness was observed after a full five-stage bleaching to dissolving pulp. Madlala et al. (2001) compared the bleaching potential of crude xylanase produced by *T. lanuginosus* SSBP strain (t_{opt} 70°C and pH_{opt} 6.5) and a commercial xylanase preparation Xyl P (60°C and 5.0) on three different types of industrial pulps (baggase-unbleached soda, hard-wood post-oxygen soda anthroquinone, and softwood post-oxygen Kraft pulp). Both the enzyme preparations were potent in bleach-boosting and reduction in consumption of ClO_2 in conventional ECF bleaching sequence. However, the effects were dependent on enzyme and pulp used. The commercial XylP was more efficient since it improved the brightness of baggase and Kraft pulps by more than 5 points and caused 30% reduction in ClO_2 consumption.

Another important application of xylanase is in the field of bioconversions. The extent of xylan hydrolysis by a mixture of xylanases from *A. fumigatus* and *H. lanuginosus* (Archana et al. 1997) was comparable to that achieved with acid hydrolysis (0.8 *N* H_2SO_4 at 121°C for 15 min.), resulting in the production of same amount of xylose (14 g of xylose from xylan). Xylanases from *M. pulchella* var. *sulfurea, T. byssochlamydoides,* and *M. albomyces* can hydrolyze xylan into xylose to an extent of 90% (Archana et al. 1997). This xylose can serve as substrate for producing protein-rich biomass (SCP) or for production of ethanol, xylitol, and organic acids via fermentation (Gilbert and Hazlewood 1993; Kuhad et al. 1997).

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Animal-feed industry is another important industrial sector where xylanases alongwith other hydrolases are directly used as feed-additive. The pre-treatment of forage crops and other biomass by xylanases improves the nutritional quality and digestibility of rumen feeds. They render easy availability of certain nutrients, absorption from cheap feed ingredients besides removing antinutritional factors (ANF) such as β-glucans, arabinoxylan, and phytic acid which adversely affect animal performance especially that of poultry (Walsh et al. 1993).

Xylanases have also found application in processing of cereals like wheat in bread making. The modification of cereal flours to enhance volume, texture, coloring, and flavouring represents a significant commercial area where potential of xylanases has been tapped. Low levels of arabinoxylanase present in bread (Kulp 1968) decreases the viscosity of pentosan solutions by fragmenting the large pentosan molecules (Schmitz et al. 1974); these alongwith starch tailings affect rheological proporties of dough and thus have a deleterious effect on bread quality and loaf volume (Kulp and Bechtel 1963). Addition of xylanases, may, sometimes have deleterious effects, causing loss in wheat flour dough water absorption and consistency, as well as changes in loaf texture (Archana et al. 1997). Xylanases markedly enhance starch recovery from wheat flour (Schmitz et al. 1974). The other applications of xylanases in food industry include, extraction of coffee and plant oil, maceration of cell walls during processing of fruits and vegetables, production of food thickeners, clarification of juices and wine, and production of xylo-oligosaccharides from xylan (Kuhad et al. 1997). The major commercial use of α-galactosidase is in hydrolysis of raffinose in the sugar beet industry (Kuhad et al. 1997). Another important use of xylanase is in the processing of oil seeds. The recovery of oil from cotton increased to 2–5% when enzyme preparation from *H. lanuginosa* was employed while addition of crude enzyme of *A. fumigatus* improved oil recovery by 4.2% from sunflower (Satyaveer and Johri 1987).

Xylanases have also found application in textile industry in the retting of flax, hemp, jute and blast. Mohiuddin (1984) reported increase in thermophilic microorganisms during the piling of jute cuttings after oil/water emulsion treatment as temperature rose to 70°C; this resulted in considerable softening of the cuttings. Cellulase-free xylanases from thermophiles with pectinolytic activity can therefore be used in softening of low-grade jute and cuttings on a large-scale (Gomes et al. 1992). Xylanase from *T. lanuginosus* produced a softer and mechanically stronger final product when applied to low quality jute fibre (Alams et al. 1994).

8 CONCLUSIONS

The immense technological applications of xylanases of thermophilic molds in various industrial sectors have made them attractive globally. They stand apart from their mesophilic counterpart by having high t_{opt} and greater thermostability. The new thermophilic molds from natural thermal environments need to be searched to find potent xylanolytic isolates. The substrate choice seems to be an important criterion for induction of a complete battery of xylanolytic enzymes. Optimization of culture conditions can significantly improve the yield. The purification techniques used are a little bit cumbersome and need to be made more simpler and quicker to achieve maximal recovery of the protein. The biochemical characterization would help us select strains, which are industrially more relevant. Moving towards the era of proteomics, the sequence matching among the xylanases of thermophilic molds, and also with their mesophilic counterparts has no longer remained beyond the scope. The sequence similarity alongwith biochemical characterisation and structure elucidation by x-ray and nuclear magnetic resonance would help us to engineer novel proteins for biotechnological applications by making use of recombinant DNA technology and site-directed mutagenesis. However, limited success on these fronts has so far been achevied. The commercialisation and biotechnological applications of xylanases of thermophilic molds is still in nascent stage and requires a major thrust. This requires fullest exploration of the existing and new gene pool of thermophilic molds by the researchers.

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Chitin Biosynthesis in Fungi

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1 INTRODUCTION

Chitin is said to be the second most abundant compound on Earth, only cellulose being more abundant. As such, chitin may be considered the most abundant nitrogenous substance in nature. Chemically, chitin may be described as an unbranched polysaccharide made of *N*-acetylglucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucose) moieties joined by β-1,4 links. Due to this type of bonding, the structural unit of the polysaccharide is not the monomer, but the disaccharide diacetylchitobiose (*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1-4)-2-amino-2-deoxy-D-glucose). Chitin chains are made of a variable number of hexose units, but normally over 1000. These chains associate among themselves by hydrogen bonding to form microfibrils made up of 20–400 molecules of the polysaccharide. Because of its crystalline arrangement, chitin is one of the most insoluble natural products, even more than cellulose itself. Scant solubilization in the order of 5% can be obtained with ethanol-containing $CaCl_2$, dimethylacetamide containing 5–9% LiCl, or *N*-methyl-2-pyrrolidone containing LiCl (Muzzarelli 1999). Based on the arrangement of the polysaccharide chains, from which their x-ray diffraction patterns depend, three different crystalline forms of chitin can be recognized in nature: α, β, and γ. The most abundant one is the α-form where chains are associated in an antiparallel form, whereas in the less-abundant β-form they are parallel; and in the rarest γ-form, two parallel chains alternate with an antiparallel one (Blackwell et al. 1978). All chitin forms behave as viscoelastic polymers, but due to the different arrangement of their sugar chains they differ in their tensile strength, which is highest for the α-form (Hepburn and Chandler 1978). Why chitin chains associate in three different crystalline forms in nature, contrasting with cellulose, where only one natural form exists, remains unknown.

An estimated amount of at least 10 gigatons of chitin are recycled in nature on a yearly basis (Muzzarelli 1999). This astonishing amount of chitin is synthesized by an immense number of organisms, all of them eukaryotes. Although a thorough discussion on the distribution of chitin is beyond the scope of this review, it is adequate to recall that the polysaccharide is present in all the animal kingdom phyla up to the Arthropods. In these, chitin represents the main structural compound (see Jeuniaux 1978). Its presence in some groups of Protista is also noticeable, as well as in some representatives of the Stramenopiles. But it is in the fungal kingdom where chitin-bearing species are more abundant, if not universal. In fungi, chitin constitutes the structural component of their cell walls, and associates with different compounds, such as proteins, lipids, other polysaccharides, and inorganic salts to form a coherent structure (see Ruiz-Herrera 1992a for a review).

Knowledge on the mechanism of chitin biosynthesis is extremely important from different points of view. In the very first place because it seems odd that we do not fully understand the biosynthesis mechanism of the most abundant nitrogenous compound in nature. Being the structural component of arthropods and fungi, its biosynthesis represents the ideal target for their specific control. The number of uses for chitin and several of its derivatives is increasing because they represent alternatives to the use of nondegradable man-made plastics. At the present time they are used in the elaboration of biodegradable packaging, surgical dressings, to preserve agricultural commodities, to

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recover proteins from waste effluents, etc. The technological aspects of chitin application have been compiled in several books (Domard et al. 1996; Muzzarelli 1977, 1993a,b; Muzzarelli and Peter 1997). Normally, the main source of commercial chitin has been shrimp and crab waste, but the use of fungi in the industrial production of chitin is an attractive possibility. And in the future it is feasible that fungal chitin synthase may be a target for genetic engineering in order to increase its efficiency.

In the present review we analyze both established and new concepts on the mechanism of chitin biosynthesis in fungi. Interested readers may consult previous reviews on the subject (Merz et al. 1999; Ruiz-Herrera 1984; Ruiz-Herrera and Martinez-Espinoza 1999; Ruiz-Herrera et al. 1992).

2 BIOCHEMICAL ASPECTS OF CHITIN BIOSYNTHESIS

2.1 General Properties of Chitin Synthases

Synthesis of chitin is catalyzed by a series of enzymes collectively denominated as chitin synthases (Chs). Chitin synthases constitute a paradoxical case since our knowledge of the genes that encode them is more thorough than the one we have on the proteins themselves. The best example of this assertion is that no active Chs has been purified yet. Some polypeptides putatively considered as the active ones, were isolated in the past, without further substantiating the original claims (see Ruiz-Herrera 1992b for a review). No wonder then that the properties of the enzymes are known from data obtained with only partially purified preparations. Particulate fractions, preparations extracted with detergents, and chitosomes, and their subunits obtained by digitonin treatment, have been the main sources of chitin synthase employed in those studies.

The kinetic properties of Chs have been described in detail in previous reviews (Merz et al. 1999; Ruiz-Herrera 1992a; Ruiz-Herrera et al. 1992). In general it can be mentioned that the universal substrate for the enzymes is the nucleotide sugar uridine diphosphate-*N*-acetylglucosamine (UDPGlcNAc). No deviation from this rule has been observed. All of the enzymes analyzed thus far require a divalent metal ion for activity. Some reported negative results were apparently due to the existence of firmly-bound residual amounts of metal ions that could be removed by dialysis against EDTA solutions (Ruiz-Herrera 1982). Since the pioneering studies of Glaser and Brown (1957) on the activity of chitin synthase from *Neurospora crassa*, it has been observed that Mg^{2+} yields the best results in most fungal preparations analyzed, closely followed by Mn^{2+}, although in some systems this was more active than the former one (Ruiz-Herrera et al. 1977). Interestingly the chitin synthase now called Chs3 from *Saccharomyces cerevisiae* requires specifically Co^{2+} for activity (Sburlati and Cabib1986).

Among the kinetic properties of the enzymes, a remarkable one is their departure from the classical Michaelian behavior. All chitin synthases display a sigmoidal response to substrate concentration, indicating a cooperative effect. Also noticeable is the apparently high K_m of the enzymes, in the millimolar range. Regarding their optimal pH and temperature, the enzymes operate around neutrality within pH limits of 5.8 and 8.0, and close to room temperature in the range of 15 and 40°C (Ruiz-Herrera et al. 1992).

Glaser and Brown (1957) were also the first investigators to describe the activation of chitin synthases by GlcNAc, which was not incorporated into the product. GlcNAc reduces sigmoidicity of enzyme kinetics (McMurrough and Bartnicki-Garcia 1971), but as noticed by Merz et al. (1999) does not abolish it, even at saturating concentrations. Because of the high *Ka* of GlcNAc, and the stimulatory effect of UDPGlcNAC, it was suggested that the sugar merely mimicked the role of the substrate at an allosteric site (McMurrough and Bartnicki-Garcia 1971; Ruiz-Herrera et al. 1977). Nevertheless, studies from Rast's group (Horsch and Rast 1993; Horsch et al. 1996) have described stereochemical requirements of the allosteric site that GlcNAC, but not UDPGlcNAc can satisfy, and confirmed the presence of an allosteric site for GlcNAc in the enzyme. To explain the source of the high concentrations of GlcNac required for chitin synthase activation (not normally present in the cytoplasm), the authors invoked the interesting theory, firstly suggested by Gooday et al. (1986), that chitin synthases operated *in vivo* associated with chitinases and diacetylchitobiase. According to this hypothesis, hydrolysis of chitin during wall growth would provide the required amounts of GlcNAc. Moreover, it was suggested that such an association would facilitate growth of the fungal cell wall (and chitinous integuments in animals) by the concerted action of lysis and synthesis.

Sequence data of chitin synthase genes (*CHS*, see below) reveal that the encoded enzymes are hydrophobic with several trans-membrane domains. Accordingly, it would be expected that the lipid environment be important for the general properties of the enzymes. This notion is supported by the inability to isolate the active enzyme outside an hydrophobic environment. Also, Arrhenius plots reveal break points for the scarce number of chitin synthases analyzed, stressing the importance of the lipid environment (reviewed in Ruiz-Herrera et al. 1992). As expected, energy of activation is lower at the higher temperature, where a transition from a solid crystalline stage to a liquid one should occur. Further evidence for the importance of the hydrophobic environment is supported by the observation that several lipids stimulate the activity of different enzyme preparations (Montgomery and Gooday 1985; Vermeulen and Wessels 1983), their activity sometimes being modified by the nature of their fatty acids.

2.2 Inhibitors of Chitin Synthases

A large number of distinct compounds have been found to inhibit chitin synthases with different degree of efficiency,

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although the mode of action of only a few of them is known. Rast et al. (2000) have made a review of chitin synthase inhibitors, and their corresponding K_i and IC_{50} values. The authors have cited the following: nucleoside peptides with K_i values from 0.2 to 7 μM: polyene macrolides; chlorinated benzenes ($K_i = 1-75$ μM); phenylphosphorodithioates ($K_i = 50$ μM); morpholines, polyaromatics, triterpenoid saponins, sterol gycosides, pseurotins ($K_i = 93$ for pseurotin A), flavonoids, dibenzazepines, aryliminodithiazoles ($K_i = 16-25$ μM); pyrimidines ($K_i = 14$ μM for 4-imidazoyl-5-phenyl-pyridine), and tetrahydropyranes ($K_i = 4-39$ μM). Most of these compounds were originally described as antifungals, and later on found to inhibit chitin synthase. On the other hand, pseurotin has no antifungal activity (Wenke et al. 1993), although its epoxy derivative does (Ando et al. 1991). The effect of polyene macrolide antibiotics on chitin synthases displays complex kinetics and high K_i (Merz et al. 1999; Rast and Bartnicki-Garcia 1981). They probably interact with sterols located in the membrane that surrounds the enzyme. In this sense it is interesting to note that our data revealed a different composition in sterols between the chitin synthase-bearing microvesicles, the chitosomes (see later), and other cell membranes in *Mucor rouxii* (Lopez-Romero et al. 1985). Novel chitin synthase inhibitors are quinolin-2-one derivatives. These are attractive noncompetitive inhibitors with K_i around 0.5 μM, which may have potential clinical use (Masabuchi et al. 2000; Sudoh et al. 2000).

An interesting competitive inhibitor of Chs described recently is pentachloronitrobenzene (PCNB; Merz et al. 1999), which was reported to be more efficient when chitin synthase was incubated in the absence of GlcNAc, at low substrate concentrations, and for short periods of incubation. The observation that the inhibitor has no structural similarity with UDPGlcNAc, and that it formed a stable complex with the enzyme that was resistant to gel filtration, led the authors to suggest that PCNB binds to an invariant tyrosine supposedly located at the active site of chitin synthases (see later).

The byproduct of the synthetic reaction, uridine diphosphate (UDP), as well as other nucleotides inhibit chitin synthases. K_i values are much higher than those described for the inhibitors mentioned above, around the value of the K_m, and kinetics of inhibition are complex (Lopez-Romero and Ruiz-Herrera 1976; Ruiz-Herrera et al. 1977), although it was suggested that UDP binds to the substrate site (Dahn et al. 1976).

The inhibitors of chitin synthase more thoroughly analyzed belong to two families of nucleoside–peptides (NP): polyoxins, produced by *Streptomyces cacoi* var. *asoensis* (Endo et al. 1970), and nikkomycins synthesized by *Streptomyces tendae* (Dahn et al. 1976). Early studies (Endo et al. 1969; Ohta et al. 1970) confirmed in many other systems (see reviews in Ruiz-Herrera 1992b; Ruiz-Herrera et al. 1992) demostrated that K_i values for these inhibitors were very low, in the μM range. These values are about three orders of magnitude smaller than the corresponding values for the K_m (see earlier). Kinetic analysis revealed that the drugs competed with the substrate at the active site of chitin synthases. Nevertheless, some data are provoking. Among them we may cite the observation that NPs do not affect initiation of glycoprotein synthesis in reactions that also utilize UDPGlcNAc as the substrate. Also interesting is the different sensitivity of chitin synthases dependent on Mg^{2+} or Co^{2+}; and the observation that the K_i for the DG42 protein, involved in the synthesis of a chitin-like oligosaccharide during *Xenopus* development is very high, *ca.* 50 μM (Semino and Robbins 1995).

2.3 Activation of Chitin Synthases

An interesting property of chitin synthases is their activation by limited proteolysis (see review in Ruiz-Herrera et al. 1992). This property was initially described for the *Saccharomyces carlsbergensis* enzyme (Keller and Cabib 1971). Further studies have revealed the almost universal occurrence of the phenomenon, although some exceptions have been cited, *e.g.* Chs3 from *S. cerevisiae* (Valdivieso et al. 1991), and chitin synthases from *Ustilago maydis* (Xoconostle-Cázares et al. 1996). Although usually referred to as "zymogen activation," the mechanism of proteolytic activation of chitin synthases is far from being understood. The main obstacle to reach a final conclusion on this matter is the absence of purified samples of the enzyme that would allow polypeptide analysis before and after proteolysis. In crude samples, all kind of phenomena may take place. In *N. crassa*, comparison of polypeptide patterns of native and protease-treated partially purified preparations of Chs, revealed the loss of a 38-kDa polypeptide, and appearance of two new ones with Mr 18 and 21 Kda (Ruiz-Herrera et al. 1987). Unfortunately, the expected mass of the active polypeptide from chitin synthases is around 100 kDa (see later), well above the observed ones. Also interesting is the observation that *Phycomyces blakesleeanus* Chs activation *in vitro* by light or trypsin is inhibited by iodoacetamide (Herrera-Estrella and Ruiz-Herrera 1983; Reyna and Ruiz-Herrera 1987), which is not a trypsin inhibitor. It has also been reported that chitin synthases from *P. blakesleeanus* and *N. crassa* are activated by calcium-calmodulin, the reaction being inhibited by the calmodulin-inhibitor trifluoperazine (Martinez-Cadena and Ruiz-Herrera 1987; Suresh and Subramanyam 1997). All these results suggest the operation of reaction cascades between the stimulus and the outcome activation, that may be brought about by a protease, or some other modifying enzyme, that, alas! has not been identified in any system.

2.4 Mechanism of Chitin Synthesis

The catalysis performed by chitin synthase has been generally considered as a simple transglycosylase reaction through which the sugar donor transfers a glycosyl unit to a growing chain of the polysaccharide. Nevertheless some evidence

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complicates this rather simple view. The first one relates to the involvement of a lipid intermediate. Most studies have revealed that a lipid intermediate of the kind involved in the synthesis of glycoproteins, does not play a role in chitin synthesis, and ruled out the existence of a high-energy intermediate (Ruiz-Herrera 1982). In *Blastocladiella emersonii*, it was described that a glucosyldiacylglycerol molecule served as an intermediate acceptor for GlcNAc, which was further transferred to the growing chitin chain (Mills and Cantino 1980). In the case of brine shrimp chitin synthase preparations, dolichol derivatives were suggested to be the initial acceptors of GlcNAc molecules (Horst 1983;1986), which were transferred in a second reaction to a protein acceptor (Horst 1989a,b). Interestingly, in the case of plants, sitosterol has been recently described to be the lipid involved in cellulose biosynthesis (Peng et al. 2002).

Merz et al. (1999) have made the interesting suggestion that chitin biosynthesis occurs in two steps with the involvement of a protein acceptor, as it happens with glycogen synthesis. They described that chitin synthase becomes activated in two distinct phases, one independent of GlcNAc, but absolutely dependent on proteolysis and UDPGlcNAC, which they interpret as a priming reaction brought about by initial glycosylation of a protein acceptor. The second one would involve further addition of glycosyl units to the acceptor formed in the first reaction to produce the long chains of the polysaccharide. We have also described evidence that activation of chitin synthase in Mucorales involves a two-step reaction, identified by use of specific inhibitors and by their differential activation by distinct metal ions (Ruiz-Herrera and Bartnicki-Garcia 1993; see Table 1). In the case of glycogen it is known that synthesis involves two different transglycosylases, one that catalyzes glycosylation of a protein named glycogenin, which in turn serves as acceptor for the second glycosylation process to give rise to glycogen (reviewed in Alonso et al. 1995; Smythe and Cohen 1991). Cellulose and β-glucan synthesis are also complex processes. In the case of cellulose, its synthesis involves a family of transglycosylases (CesA proteins), a cellulase (Korrigan), and sitosterol as lipid carrier (see Peng et al. 2002; Read and Bacic 2002 for recent reviews). Although no protein acceptor has been described, the process involves the synthesis of an initial cellodextrin using sitosterol as acceptor (see earlier). As occurs with glycogen, during the synthesis of β-glucans we have described evidence that the process involves glycosylation of protein acceptors, whose chains are extended at a latter period (Ruiz-Herrera and Larriba 1995).

It must be stressed that during chitin synthesis two different processes occur, transglycosylation *sensu stricto*, and fibrilogenesis, to give rise, not to a soluble compound, but to a stiff microfibril made by the association of *ca*. 20–400 sugar chains each containing about 2000 sugar units (Calvo-Mendez and Ruiz-Herrera 1987). Different lines of evidence indicate that the processes of transglycosylation and fibrilogenesis are not simultaneous, but are separated by a time gap: (a) Sugar chains in α chitin are anti-parallel, and synthesis occurs by sugar addition to the nonreducing end only. Accordingly, fibril formation must await chain termination in order to proceed; (b) Nascent chitin, in contrast to the crystalline form is very sensitive to chitinase (Lopez-Romero et al. 1982), and chitin deacetylase (Calvo-Mendez and Ruiz-Herrera 1987; Davis and Bartnicki-Garcia 1984); (c) Chitin synthesized in the presence of Calcofluor white is very sensitive to chitinase and noncrystalline, but upon drying becomes crystalline and resistant to enzymatic hydrolysis (Vermeulen and Wessels 1986).

3 CYTOLOGICAL ASPECTS OF CHITIN BIOSYNTHESIS

Data on chitin biosynthesis obtained *in vitro* are clearly insufficient to explain diverse physiological aspects of the process such as the nature of the product, its location in the living cell, and the asymmetry of the process, as detailed later. Chitin precursors are synthesized in the cytoplasm, and chitin is accumulated in the cell wall. Several pieces of evidence have led to establish the concept that in fungi, Chs follows the normal exocytic route and accumulates inside the cell. Relevant among these we may cite the following: (a) Chitin synthesis by toluene-permeabilized cells of *M. rouxii* occurs in the cytoplasm, but not in the plasma membrane (Sentandreu et al. 1984); (b) Electron microscopic immunochemical studies evidence that chitin synthase is localized in apical microvesicles (chitosomes) in *Neurospora crassa* (Sietsma 1996); (c) Immunocytochemical data show that chitin synthase 3 (Chs3) from *S. cerevisiae* locates in the Golgi, and post-Golgi vesicles (chitosomes) before reaching the cell surface (see later).

A vast amount of evidence has accumulated demonstrating that chitosomes are the specialized type of microvesicles

Table 1 Activation of chitin synthase in *Mucor rouxii* is a two-step process

Parameter	Step 1	Step 2
Activator	Exogenous acid protease	Unknown
Inhibitors	Pepstatin A	PMSF, dichloroisocumarine
UDPGlcNAc	Required	Substrate
Efficiency of divalent metals	Ca > Mn > Co > Mg	Mg > Mn > Co > Ca

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where chitin synthase is accumulated in fungi, and are afterwards targeted to the sites at the cell surface where chitin synthesis takes place. Chitosomes have been thoroughly analyzed, and found to contain unique chemical and physical properties (see reviews in Bartnicki-Garcia et al. 1979; Bracker et al. 1976; Ruiz-Herrera 1984; Ruiz-Herrera 1992c; Ruiz-Herrera and Martinez-Espinoza 1999; and Figure 1). They measure about 40–70 nm in diameter, and are surrounded by a 6.5–7.0 nm thick membrane. They are made of two parts of protein of a unique composition, and one part of lipids, with a composition different to the rest of the cell membranes. Chitosomes synthesize chitin microfibrils *in vitro*, essentially identical to those found in the cell wall, and can be dissociated into active subunits with an Mr of 500,000 Da that have the property to reassemble into chitosome-like aggregates.

Chitin synthase-containing vesicles are not exclusive of fungi; they have also been identified in different arthropods

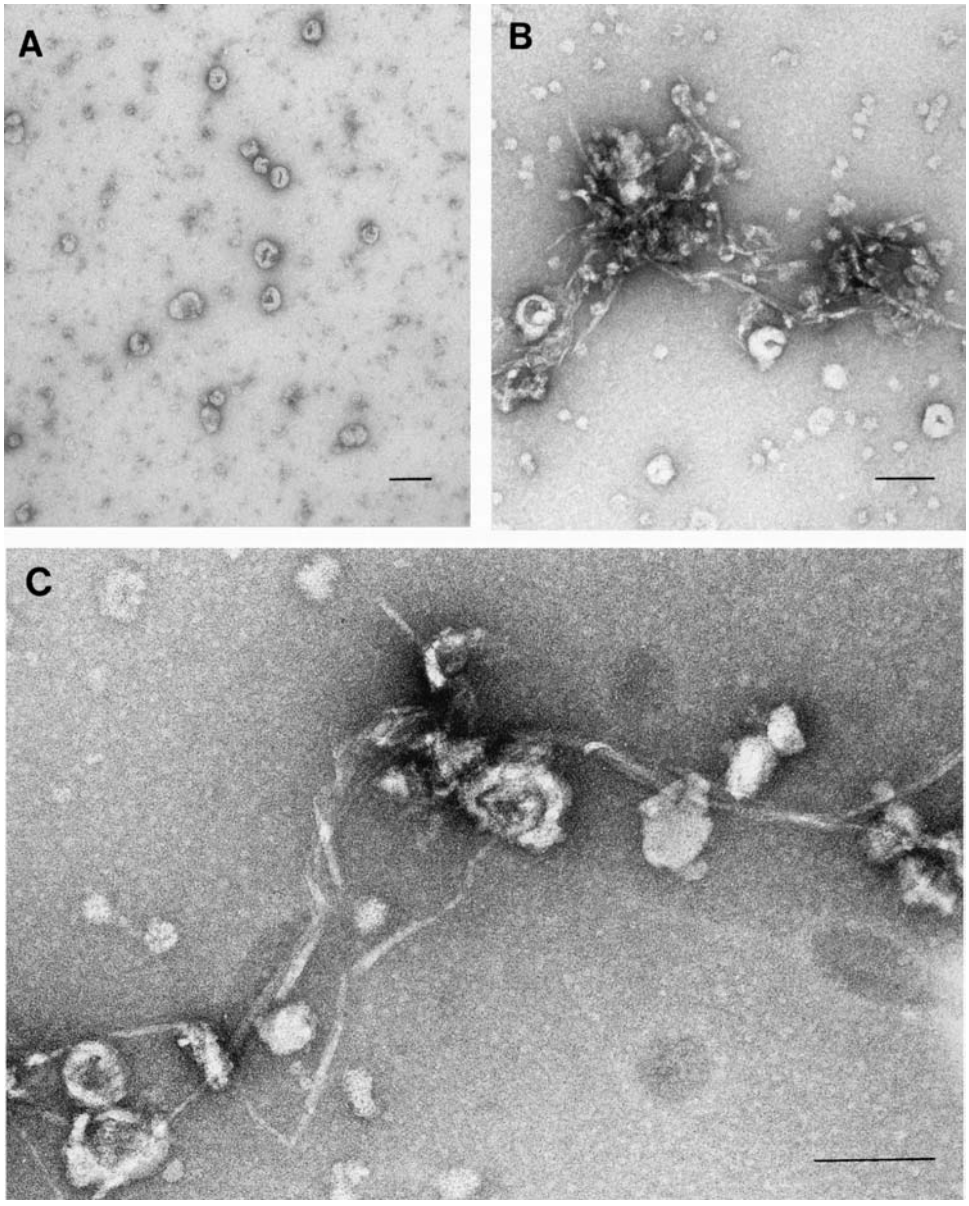

Figure 1 Chitosomes and synthesis of chitin microfibril *in vitro*. A. Chitosomes isolated from *S. cerevisiae*. B. Chitin microfibrils synthesized *in vitro* by chitosomes isolated from *S. cerevisiae*. C. Detail of chitin microfibrils synthesized by chitosomes isolated from *M. rouxii*. Photographs by C. Bracker, S. Bartnicki-Garcia, and J. Ruiz-Herrera. Magnification bars, 100 nm.

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and protozoa, and intracellular synthesis of the polymer is well documented in some of these organisms (Cohen 1982; Horst and Walker 1993; Mulisch et al. 1993). In fungi most authors are inclined to think that synthesis of chitin occurs in the plasmalemma, although no definite evidence has been provided to exclude the possibility that the polysaccharide synthesis initiates within the chitosome before it fuses with the plasma membrane.

Study of the process of intracellular mobilization of chitin synthase by the exocytic route has been mostly circumscribed to Chs3 from *S. cerevisiae*. These studies have been facilitated by the analysis of several *CHS* genes (*CHS4* to *CHS7*) that do not code for Chs catalytic polypeptides, but whose mutation leads to a reduction in the amount of chitin in the cells (see review in Roncero et al. 2001). Strategies used for the identification of these genes were based on either the isolation of calcofluor white-resistant mutants (Roncero et al. 1988), or defective incorporation of [^{3}H]-GlcNAc into chitin *in vivo* (Bulawa 1992). The best known of these genes is *CHS5* that codes for a protein that colocalizes with Chs3 in the yeast cell in Golgi and in post-Golgi vesicles (chitosomes) (Santos and Snyder 1997). *Chs5* mutants are affected in Chs3 polarization, and fail to localize Chs3 at the growing sites of the cell where chitin synthesis occurs. These data strongly suggest that Chs5p is involved in the correct cellular localization of Chs3. Nevertheless it appears that Chs5p is involved in other processes as well; Fus1p and Fus2p that are required for cell fusion during mating, also display the same localization as Chs5p, and *Chs5* mutants are affected in this process. *CHS4* codes for a protein also required for the correct localization and activation of Chs3 (Ono et al. 2000; Trilla et al. 1997). Apparently the protein is involved in the attachment of Chs3 to the neck of budding cells by an interaction with Bin4 (DeMarini et al. 1997). Another gene product apparently involved in the correct mobilization of Chs3 is *Chs6*. *chs6* mutants contain reduced amounts of chitin, and accumulate the enzyme inside chitosomes (Ziman et al. 1998). Finally we must mention Chs7p, an ER-resident protein which has been suggested to operate as a chaperone for Chs3, as evidenced by the observation that *chs7* mutants accumulate inactive Chs3 within the ER (Trilla et al. 1999).

After chitin reaches the extracellular space it may be subjected to different modifications, bind through covalent or noncovalent links to many different compounds, and give rise to organized composites that are responsible for the protection of the cell. In fungi this composite is the cell wall, whereas in other organisms it is the exoskeleton. A thorough discussion of these important aspects is beyond the scope of this manuscript; interested readers may find a review in Ruiz-Herrera and Martinez-Espinoza (1999). As a brief summary we may point out that the synthetic process from the soluble substrate to the final cell wall involves the following steps: synthesis of sugar chains, chemical modifications of certain amounts of the polymer, microfibril formation, establishment of covalent and noncovalent associations, and finally organization of the corresponding composite.

4 GENETIC CONTROL OF CHITIN SYNTHASES

4.1 Genes Coding for Chitin Synthases in Fungi

One important advance in the study of chitin synthesis was the cloning of the genes (*CHS*) that encode the active polypeptides of chitin synthases. The first one to be cloned was *CHS1* from *S. cerevisiae* by complementation of mutants affected in the *in vitro* synthesis of the polysaccharide (Bulawa et al. 1986). Two surprising results were obtained during the analysis of the gene. The first one was the size (130 kDa) of the encoded polypeptide (Chs1), a value quite in excess of the previously suggested active polypeptides (see above). The second one was the observation that null mutants obtained by gene disruption did not display any significant phenotypic alteration (Bulawa et al. 1986). This result suggested the existence of an additional chitin synthase in the fungus, a suggestion that proved to be true when the gene (now called *CHS2*), encoding a second chitin synthase (Chs2) with a high level of similarity to Chs1 was cloned (Silverman et al. 1988). Although initially it was published that *chs1/chs2* mutants were nonviable, further studies revealed that the double mutation was not lethal, and that ascospore germination occurred almost normally if incubated under adequate conditions (Bulawa and Osmond 1990). This unexpected result led to the search for still another chitin synthase, whose coding gene was finally cloned independently by use of two different approaches (Bulawa 1992; Valdivieso et al. 1991). Later on, the observed similarities in the protein sequence of the different chitin synthases allowed the design of degenerate oligonucleotides for the identification of *CHS* gene fragments from a large number of fungi by PCR (Bowen et al. 1992).

Studies carried out mainly in *S. cerevisiae*, have identified other genes also involved in chitin biosynthesis, but apparently not coding for the catalytic polypeptides as revealed by sequence comparison. These genes have received different names, but most of them are known as *CHS* genes 4 and on. Their role in the regulation and cellular location of chitin synthase was discussed earlier.

4.2 *CHS* Gene Multiplicity in Fungi

Studies with a large number of fungal species have demonstrated that the presence of multiple genes coding for chitin synthase is not unique for *Saccharomyces*. Accordingly, the Zygomycete *P. blakesleeanus* contains 10 *CHS* genes, the Ascomycete *Aspergillus fumigatus* 7, and the Basidiomycete *Ustilago maydis* 6. This demonstrates that *CHS* gene multiplicity is a general phenomenon, and not restricted to a single group of fungi (see review by Ruiz-Herrera et al. 2001). Several hypotheses have been suggested to explain the benefits of having different chitin synthases. An attractive one is the possibility that the different enzymes play different roles in the physiology of the organism. Taking into

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consideration the relative distribution of chitin in the cell wall of *S. cerevisiae chs* mutants (Shaw et al. 1991), it was suggested that each enzyme played a selective role. Chs3 was considered the most important one, and would be involved in the synthesis of the chitin ring during bud emergence, of chitosan in the spore wall, and of most of the wall chitin of the yeast. Chs2 would be involved in the formation of the primary septum that separates mother and daughter cells during yeast budding; whereas only a minor role in wall repair during cell division was suggested for Chs1, the most active enzyme *in vitro*. However since even double mutants are viable, i.e., they can perform all these processes [only the triple mutation is lethal (Shaw et al. 1991)], it appears that there is no absolute distribution of functions, and that, on the other hand, there is an overlap in the function of these enzymes.

4.3 Phenotypic Alterations in *Chs* Mutants

We have distinguished five different degrees in the severity of phenotypic alterations in fungal *chs* mutants: (a) not significant; (b) minor alterations; (c) significant alterations; (d) severe alterations; and (e) lethality (Ruiz-Herrera et al. 2001). The most common phenotypes of *chs* mutants are either not significant or display of only minor alterations, even in cases where two Chs are altered. Additionally there is no correlation between the severity of the phenotypic alterations, and the class of the enzyme (see later) affected in the mutants. As described above most chitin in the wall of *S. cerevisiae* is the product of Chs3, a class IV enzyme (Bulawa 1992), whereas a Class I enzyme (Chs1) is responsible for this role in *N. crassa*, (Yarden and Yanofsky 1991). It may also be noticed that severe phenotypic alterations as result of the loss of a single Chs may occur during the whole life cycle of the fungus, or at specific phases of growth. Accordingly, *N. crassa chs1* mutants are grossly affected in their vegetative growth (Yarden and Yanofsky 1991), but *chs6* mutants from *U. maydis* grow almost normally, but become avirulent (A. Garcerá et al. in preparation). Only one case of an essential *CHS* gene is known. Microconidia from *N. crassa chs3* mutants are nonviable although the fungus contains four *CHS* genes (Beth-Din and Yarden 2000).

Taking into consideration that selection tends to conserve beneficial traits and eliminate redundancy during evolution, unless it proves useful for competence, one may conclude that the existence of multiple chitin synthases in fungi must be beneficial for their survival. Example of this assertion is the behavior of a *U. maydis chs3* mutant that displays only minor phenotypic alterations (Xoconostle-Cázares et al. 1996). If this mutant is inoculated together with a wild-type strain in synthetic medium, both grow at about the same rate. But if it is inoculated into maize competing with the wild-type strain, it is almost completely eliminated from the plant (Table 2, J. Ruiz-Herrera and C. Leon, unpublished). This result indicates that loss of even a single, and apparently redundant chitin synthase may affect survival of a fungus in a competing natural environment. We have previously suggested that conservation of Chs multiplicity probably represents a

Table 2 Competition between wild-type and Δ*chs3::hpt* strains of *Ustilago maydis in vitro* and *in vivo*

A. *In vitro*

About the same numbers of cells from both strains were inoculated in synthetic medium and incubated at 28°C with shaking. After 24 and 48 h, aliquots were taken, diluted, and inoculated on plates of synthetic medium with or without 250μg hygromycin per ml, to calculate total, and hygromycin resistant (i.e., *chs3*) numbers of cells

	Number of cells ($\times 10^{-6}$ per ml)		Ratio
Time of incubation (h)	Wild-type	Δ*chs3::htp*	wt/*chs3*
0	1.28	1.40	0.90
24	10.44	10.26	1.01
48	16.39	14.25	1.10

B. *In vivo*

About equal number of cells of strains wt a1b1, Δ*chs3::hpt* a1b1, and wt a2b2 were mixed and inoculated into 11 maize plantules. After three weeks, the tumors that developed in the plants were dissected, and teliospores were isolated and inoculated on plates of synthetic medium to allow their germination. Sporidia obtained at this stage were diluted and inoculated on plates of synthetic medium with or without 250 μg hygromycin per ml, to calculate total, and hygromycin resistant (i.e., *chs3*) numbers of cells

	Number of colonies		Ratio
Time of incubation on plates (h)	Wild-type	Δ*chs3::htp*	wt/*chs3*
96	1108	1	$>1/10^{-4}$

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fail-safe mechanism that allows fungal survival in hostile environments (Chavez-Ontiveros et al. 2000). A similar situation exists in plants. It is known that plants contain a whole family of *CesA* genes encoding the active polypeptides for cellulose synthesis (see, for a review, Delmer 1999). Analysis of gene expression in maize demonstrated that different *CesA* genes are expressed in all organs. Although a differential expression of gene families was detected in cells involved in primary or secondary cell wall formation, mutation in a single gene did not lead to a significant phenotype alteration (Holland et al. 2000). These observations, as in fungi, indicate redundancy of function.

5 REGULATION OF CHITIN SYNTHASES

Important information on the mechanisms of Chs regulation has been uncovered, no matter that some confusing, and even conflicting data exist among the different species studied. In general we can recall that, as indicated earlier, most chitin synthases are activated *in vitro* by controlled proteolysis (see review in Ruiz-Herrera et al. 1992). Independently of this phenomenon, it is apparent that in general most chitin synthases are under some sort of regulation *in vivo* (for a review see Ruiz-Herrera et al. 2001). Thus we may conclude that the final activity of Chs *in vivo* depends on complex interactions between transcriptional and post-transcriptional regulatory mechanisms, whose net effects probably depend on the system and the environmental conditions (Ruiz-Herrera et al. 2001).

One way to approach the possible existence of transcriptional regulation of *CHS* genes is the analysis of potential binding motifs for transcriptional regulators in the upstream region of the genes. Such a study (Ruiz-Herrera et al. 2001) has indicated the existence of canonical sites for the binding of different transcription factors, among them those responding to heat shock, stress, pH, nitrogen deprivation, phosphate levels, etc. Interestingly, different genes from the same species displayed both specific and common motifs, supporting their activation under different conditions, but also the existence of an overlap in regulation, in agreement with mutational analysis. These theoretical results are supported by the single system exhaustively analyzed in this regard. A transcript corresponding to *CHS3* from *Exophiala dermatitidis* accumulated to high levels when the fungus was grown at high temperature, at an acidic pH, or under nitrogen limitation (Wang and Szaniszlo 2000). In agreement with these results, potential binding sites for the following transcription factors were identified in the 5′ upstream region of the gene: Stre01 involved in stress reactions including heat shock; PacC, involved in gene regulation by pH, and Nit2 (AreA) that positively regulates gene expression by nitrogen deprivation. Besides this example, there are a significant number of reports describing that throughout their life cycle, morphogenetic events, or as a result of changes in the environmental conditions, important changes occur in the levels of *CHS* transcripts in different fungi. The first one to be reported was in the levels of CaChs2 mRNA occurring during the dimorphic transition of *C. albicans* (Chen-Wu et al. 1992). During the yeast-to-mycelium dimorphic transition of *U. maydis*, we have detected higher levels of transcripts of different *CHS* genes (Xoconostle-Cázares et al. 1996;1997). In *Mucor circinelloides CHS1* transcript accumulated in the mycelial, but not in the yeast forms (Lopez-Matas et al. 2000).

In other systems, environmental conditions have been found to affect the levels of chitin synthase activity, probably at the post-transcriptional level. The following are some examples. In results that agreed with its *in vivo* response, Chs activity from *Phycomyces* sporangiophores was increased when they were illuminated by blue light (Herrera-Estrella and Ruiz-Herrera 1983). This phenomenon apparently involves a mechanism dependent on calcium-calmodulin (Martinez-Cadena and Ruiz-Herrera 1987; Ruiz-Herrera et al. 1990), as it occurs in *N. crassa* (Suresh and Subramanyam 1997). Similarly, osmotic stress induced higher levels of chitin synthase activity in *Benjaminiella poitrasii* (Deshpande et al. 1997).

Likewise, alterations in cell wall synthesis may affect Chs regulation, probably as the result of compensatory mechanisms, not well understood yet. *S. cerevisiae* mutants in *GGP1/GAS1*, a gene coding for a GPI protein, contained reduced amounts of β-glucans, but higher concentrations of chitin (Popolo et al. 1997). Similarly, mutation in *FKS1*, the gene coding for β-glucan synthase of the yeast (Garcia-Rodriguez et al. 2000) led to an increase in the content of wall chitin. It was suggested that these changes were the result of alterations in the localization of Chs3 and of Chs4, a regulator of chitin synthase activity (see earlier). To explain why *CHS* transcripts levels were severely reduced, but chitin levels were only moderately affected in mutants of *S. cerevisiae* containing the negative transcription regulator of *CHS* genes Knr4, Martin et al. (1999) suggested that in normal conditions most of the chitin synthase accumulates in an inactive form inside chitosomes. These two examples are indicative of a structural regulation of chitin synthase.

6 CLASSIFICATION OF CHITIN SYNTHASES

Sequence comparison of the first cloned *CHS* genes revealed strong similarities between them. These results were further confirmed by a systematic amplification of a number of fungal *CHS* gene fragments by PCR (Bowen et al. 1992). These results led to an initial classification of Chs into three groups, which were later extended to five when more PCR fragments and complete sequences were obtained (Specht et al. 1996; Xoconostle-Cazares et al. 1997). Recently we made a thorough analysis of the classification of Chs that provided interesting conclusions (Ruiz-Herrera et al. 2002). The results evidenced

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that animal Chs departed from fungal Chs early in evolution, and that these separated into two distinct divisions, one radiating into three different classes, and the other into two classes (Figure 2). Further division into subclasses is apparent in some instances. Class II, for e.g., may be subdivided into two groups that included on one side Chs from Basidiomycetes and Ascomycetes, and Zygomycete enzymes on the other one. Interestingly, no Class III Chs representatives were found among Zygomycetes, even though *P. blakesleeanus* contains the largest number of *CHS* genes (10) among fungi (Miyazaki and Ootaki 1997). The significance of this observation is discussed below.

7 EVOLUTION OF CHITIN SYNTHASES

It must be recalled that the presence of chitin is restricted to eukaryotes, and that the polysaccharide is present in members of all the "crown kingdoms," with the exception of plants. Accordingly chitin synthase is probably an ancient enzyme that may have appeared once plants had diverged from the eukaryotic groups, i.e., approximately one billion years ago. Its precursor may have been the ancestor of modern β-glycosyltransferases. These display certain common motifs with Chs (see later). In accordance with this hypothesis, it may be cited that analysis of a large number of enzyme sequences has

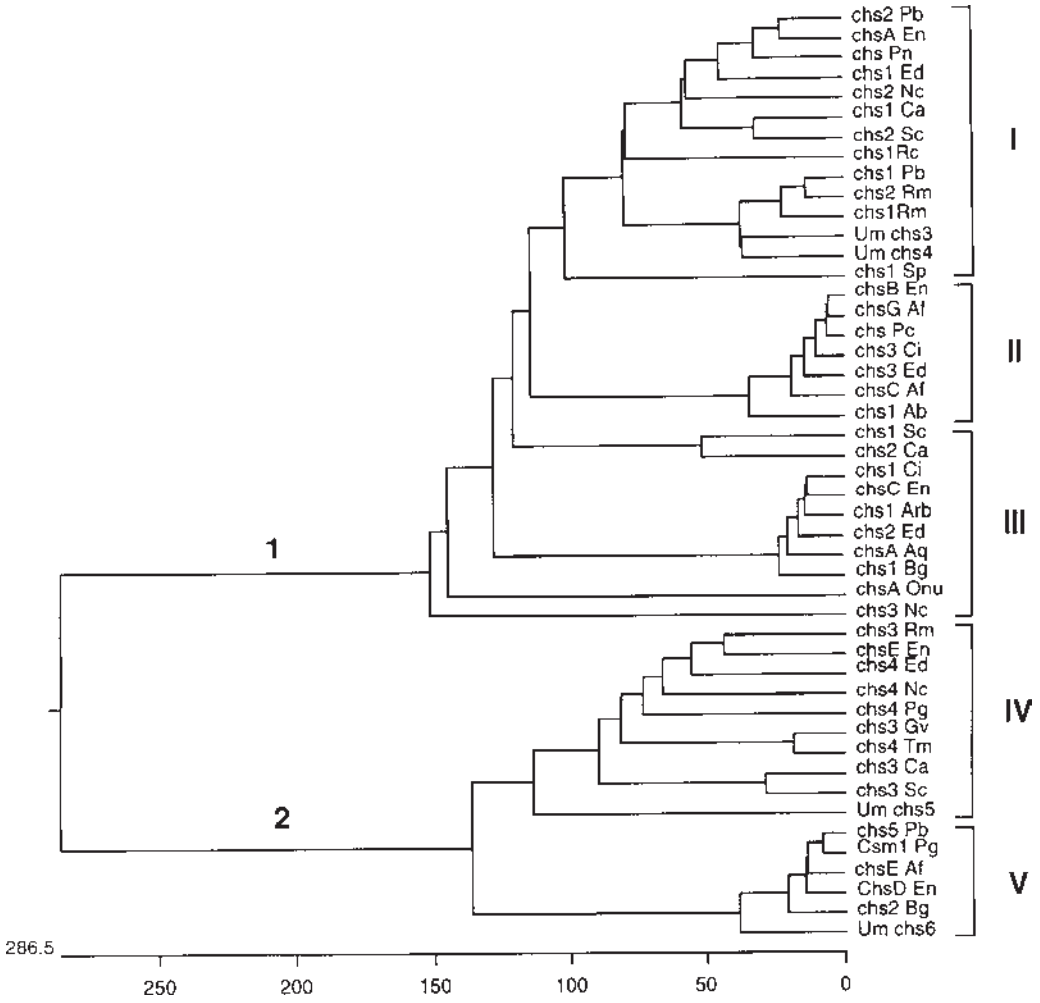

Figure 2 Maximal parsimony dendrogram grouping chitin synthases from fungi with their corresponding classification into divisions (1 and 2) and classes (I–V). Ab, *Agaricus bisporus*; Arb, *Arthroderma benhamiae*; Af, *Aspergillus fumigatus*; An, *Aspergillus niger*; Aq, *Ampelomyces quisqualis*; Bg, *Blumeria graminis*; Ca, *Candida albicans*; Ci, *Coccidioides immitis*; Ed, *Exophiala dermatitidis*; En, *Emericella nidulans*; Gv, *Glomus versiforme*; Nc, *Neurospora crassa*; Onu, *Ophiostoma novo-ulmi*; Pb, *Paracoccidioides brasiliensis*; Pc *Penicillium chrysogenum*; Pg, *Pyricularia grisea*; Pn, *Phaeosphaeria nodorum*; Rm, *Rhizopus microsporum*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tm, *Tuber magnatum*; Um, *Ustilago maydis*. (Ruiz-Herrera et al. 2001, with permission).

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Table 3 Consensi among chitin synthases. Consensi are classified according to Class, Division, Kingdom, and all Chs analyzed. Consensi are numbered according to the relative position along the protein, starting at the amino termini. Division 1, 1–26; Division 2, 31–45; Oomycetes, 51–56; animals, 61–74. *Chs from *S. monoica*

CLASS I

1.
2. FF(T/A)K (D/F)MRYXA(A/V)TC(D/G/F)F
3. V(Y/I)TMYNE(D/E)
4.
5. WKKIVVL(V/F)(V/I)SDGR(A/T/S)KIN

6. YQD(D/E)XG(I/L)AK
7.
8. FCLKE(K/T/Q)N(Q/A)KKINSHRW
9.
10. L(I/L)D(A/C)GT
11. S(M/L)YXLW
12. (C/Y)(A/G)G(A/G)CGE(K/TR)
13.
14. NPLV(A/V)X(Q/N)FEYK(M/L)SNLLDK(P/T)(L/T)ES
(A/S/N)FGF(S/T)VLP(G/A)AF(S/C)AYR
15
16.
17. YFXGE
18. F(T/S)(A/S)NMYLAEDRILCF(E/D)(L/V)Y

19. (E/S)TDVP
20. E(F/L)(I/V)(L/S)QRRRWLNGS(F/L)(F/A)A
21
22.
23.
24. (D/E/Q)PWHM(F/V/L)T(S/C)
25. LN(I/V)YA(F/L)CN(T/I/V)(H/D)D(G/L)(T/S)WGTKG
26.

CLASS II

1. E(T/A/S)X(M/L)(R/T)Y(T/S)(A/V/C)TC(V/S)D(Q/F)P
TMYNE(D/N)

WXK(V/I)(K/Q)S)VX(I/V)(I/V)(S/A)D
GRX(K/A/V)(V/I)(H/N)

AH(V/I)(Y/F)E(Y/L/I)E(T/A)(C/V/L)S(D/N)
FC(L/M)KEXNXKK(I/L)NSH(R/L)W(F/L)E(N/Q)AF

I(L/V/I)(D/E)(C/V/A)GT
(S/A)(L/I/Y)XLW,XFD

NPLVA(S/T/A)QNFEYK(M/L)SNILOK(P/T)(L/T/V)
ES(V/L)(F/M)G(Y/I/F)(L/S/I)LPGA(L/F)SAYR

GXGPL
YFXGE
F(T/E)(A/S)NMYLAEDRILC(W/Y/F)(E/D)(L/V/A)(A/S)K

(S/V/I)TDVP
F(F/L)(I/V)(S/L/I)QRRRW(L/I)N(G/Q)X(F/M)FA

CLASS III

(D/E)YPV?(S/T/I/A/P)I(Q/L)NA
FF(T/S)(I/M/R)YTAAT(C/V) (D/P)
MYNR(H/D)TE(L/R/I) LLA T(Y/S)YNE(D/E)K
RTLHGVM(Q/L)N(I/V)RDI
WQKI(V/T)(V/C/A)L(V/G)(E/Y)DG(T/L)
(D/E)PC(E)K
YQDG/VMK
TI(V/L)AHISEYTTQLSV
F(C/V/L)K(Q/A)(K/Q)N(S/R/Q)KKINSHRWLFNA
LNPF(V/I)K"
I(L/I)DAGTKPG(S/P/H)KS(L/I)

LGG(A/S/C)CGEIHAM

NPLVAAQNFEYK(I/M)SNII DKPLIS(S/A)FGYV
SVLPGAFSAYR
PLEQVFEHG

LG(K/P)QG(E/Y)GMNIF
(K/I)KNMFLAEDRILCFELVAKA
F(I/D)VPE
E(F/L)(S/G)QRRRWLNGSFAA
RMFF
WF(S/A)LA
LQF(I/V)LALGNRPK
YXDPWRM(M/I)(F/L)(T/S)S
L(M/N)(V/I)YAF(S/C)N(W/L)HDVSWGTKGSDK
FRT(R/S)LV

DIVISION 1

2. E F (T/ASK) X (M/L) (R/T) Y X A (A/V/C) T (C/V/S)
3. T (M/Y/S) Y N X (E/E/N)
5. W X K (I/V) X V X X (V/I) X D G
8. F (C/V) (L/M) K (E/Q/A) X N X K K (I/L) N S H (R/L) W
10. (L/I) (L/I/V) (D/E) (A/V/C) G T
14. N P L V (A/Y) X Q N F E Y K (M/L) S N I L D K (P/T) (L/TV) E S X (F/M) G (Y/F/H) (I/V) (S/T) V L P (G/A) A (F/L) (S/C) A Y R
18. N M (Y/F) I. A E D R I L C (F/W/Y) (E/D) (L/V) (V/A)
19. T D V P
20. E (F/L) (I/V) X Q R R R W (L/I) N (G/Q) X (F/I/M) (F/A) A

CLASS IV

31. V(G/A)(I/Y)(L/I)TFGF
32. TLDS(L/I)(A/S)XTDYP(N/S/H)K
33
34. KRHN(M/K)(A/C)(K/Q)(I/V)Y(A/L)G(F/Y)Y
35. QQR(V/I)P(M/I)
36. K(P/A)GNRGKRDSQ
37. LXVIDATDKVIP(Y)PD(S/A)L
38. MGLCGFTKR(A/S)NK
39. QVEFY(F/Y)(D/V)SHNXXK(A/S)FE(S/A)XFG(G/S)VTCLING(T/)

40 TLHXKNLLLLGEDR(Y/F)(L/S/T)LML

41 TFP(K/T)RK
42. LLSQ(R/G)RRWINST(V/I)HNL
43 DLCGTFCFSM(Q/R)F
44
45 DDFSWGXTR

CLASS V

Y(S/D)I(R/K)RKL

PGNRKRDSQ
DADTK(V/R)(V/I)
CGLI
QV(Y/F)EY(Y/F)SH(N/H)(L/I)XKAFES(V/L)
FG(S/G)(V/I)TCLPG(C/R)E(T/S)(M/L)YRI
K(D/E)YTLHXKNLLG(I/S)LGEDR(Y/F)(L/T/S)
T(L/I)

(L/M)SQRRRWINST VHNL

GL(Q/P)AI
Y(S/A)FWH(M/F)DDF(S/T)WG(N/E)TR

DIVISION 2

36. (P/A) G N R G K R D S Q
37. D A D T K (V/R)
38. C G E T
39. Q V (Y/F) E Y (Y/F) (I/V) S H (N/H) X X K (A/S) F E (S/A) X F G (G/S) (V/I) T C L P G (C/R) F
40. T L H X K N L L G E D R (Y/F)E(L/S/T)LML
42. S Q (R/G) R R W I N S T (V/I) H N L
45. D D F (S/T) W G X T R

ALL FUNGI

I. (14/39) Q X X E Y.....L P (G/A)
II. (18/40) L (A/G) E D R X L
III. (20/42) Q (R/G) R R W (L/I) N

OOMYCETES

51 V(V/A)HVSDGRTKA(N/S)K
52 PP(T/L)QVX(E/Y)A LKP(N/H)N(G/A)GKL(N/D)SH(L/E)W(Y/F)E(N/D)AFEQ
53 PXYTVL(L/V)DVGT(M/I)P

54 QHPEY*
55 LAEDRILC*
56 QARRW*

ANIMALS

61 CATMW(E/V)T
62 LKSI
63 D(P/D)(D/A)(Y/R)V (E/R/D)(E/L)E(T/A/V)I (I/T/V)F(F/L)(D/E)(A/V)XF'WIF
65 HLKDK
66 RH(R/K)KRWSQVMY(M/L)YYLLG
67 NT(Y/F)(I/L)(T/A)(I/D)GD
68 G(A/C)AGRIHP(V/I)G(S/TE)G(P/I)M(V/I)WYQ
69 QXFEYA(G/S)HW(L/F)QKA(T/A)EH(M/V/I)(I/F)GCV1C(S/A/P)FGCFS(L/P)FR
70 AHRL.IH(V/F)VQ(Y/C)DQGEIRWI (C'SSTLLL
71 EF(Y/F)NQRRRW(V/T)P(T/S)IAN
72. DLLXI(X/A/Y)KR
73. LHPQE
74. WGTRE

ALL CHITIN SYNTHASES

I. (14/39/54/69) Q X X E Y
II. (18/40/55/70) E D R X L
III. (20/42/56/71) Q X R R R W

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provided evidence that animal and fungal kingdoms evolved as separate groups after splitting from the plant lineage (Doolittle et al. 1996). These results are in agreement with the separation of animal Chs as a compact group with no apparent subdivisions (Ruiz-Herrera et al. 2002). Regarding Chs from Stramenopiles, the only enzymes analyzed thus far are restricted to a few Oomycetes. These constitute a single family of Chs, with similarities to fungal division 1 Chs (Ruiz-Herrera et al. 2002). As alternative possibilities to explain this similarity, we have suggested a case of convergent evolution, or the occurrence of a late process of horizontal gene transfer. These processes are not common in fungi, but their existence has been substantiated (Rosewich and Kistlwer 2000).

An important observation in our analysis of the relationships among Chs was the lack of correlation between fungal and chitin synthase taxonomies. Accordingly, representatives of both Chs divisions and five classes are present in members of all true fungi. Conceivably, separation of the fungal groups had been preceded by diversification of chitin synthases from a common ancestor (Ruiz-Herrera et al. 2002). A similar conclusion has been drawn from analysis of cellulose synthase *CesA* genes, in that they evolved into classes prior to plant divergence into monocotyledons and dicotyledons (Holland et al. 2000).

It is most probable that separation into divisions set the initiation of fungal Chs diversification. By comparison of the conserved motifs in Chs (Table 3), we noticed that they are restricted to a short peptide stretch in the different classes of fungal Chs, located close to the carboxy termini in division 2 Chs. Based on these results we suggested that the event that gave rise to separation of Chs into divisions was the acquisition or loss of a long amino terminus by a new family of Chs (Ruiz-Herrera et al. 2002). This event should have been followed by separation of Chs into classes. Our data have revealed that Chs from class III have the highest level of similarity among the different classes, suggesting a more recent origin (Ruiz-Herrera et al. 2002). This result is further supported by the presence of common motifs between the different Chs classes (Table 3). Accordingly, classes I and II share all of their common motifs, except one, specific for class II; whereas class III enzymes contain specific motifs not shared with classes I and II (Table 3).

Diversification of Chs into divisions and classes must have occurred after the fungi first splitted from the eukaryotic lineage, (an event that apparently occurred approximately one billion years ago), but before they gave rise to the different taxa. Analyses of 18S ribosomal RNA gene sequence, 5S sequences, biochemical data and calibration with fossil records support the idea of a former division of fungi into the Ascomycota–Basidiomycota and Zygomycota–Chytridiomycota lineages about 600 Ma ago (Berbee and Taylor 1992; Redecker et al. 2000; Walker 1985).

A contradictory observation regards to the absence of class III Chs in Zygomycetes, as described earlier. Since members of this fungal group contain Chs belonging to both divisions, and separation into division preceded class separation, it is likely that the precursor of that group of enzymes was lost in the Zygomycete ancestor during evolution. The argument of selective loss of *CHS* genes can also explain why not all fungal species contain representatives of all Chs groups. In contrast, the presence of two or more Chs of the same class in a single fungal species probably represents late gene duplication events.

8 ON THE STRUCTURE OF THE ACTIVE SITE OF CHITIN SYNTHASE

It is unfortunate that no chitin synthase has been purified. This fact has precluded the use of techniques that would have otherwise provided information on the structure of the active site of the enzyme. The only approaches that have been followed to gain some insight on this matter have been comparisons with other β-glucosyl transferases, and the use of model systems. The results are still fragmentary, although some interesting concepts have been established. We have used as an approach the exhaustive comparison of chitin synthases from fungi, animals, and oomycetes in order to identify the conserved amino acids and motifs characteristic of each class, division, and taxonomic group (Ruiz-Herrera et al. 2002). We have also used specific programs to determine their relative location (Ruiz-Herrera et al. 2002). Table 3 shows the motifs conserved in the different groups of chitin synthases, and the motifs conserved in all Chs.

In relation to the topology of the enzyme, our analyses showed the existence of three transmembrane helices in the conserved region of all Chs, two of them located towards the amino termini, and one towards the carboxy termini. The QXRRW motif was located in a flexible region at the surface of a transmembrane helix surrounded by hydrophilic stretches (Ruiz-Herrera et al. 2002).

Different experimental approaches have suggested a role for the QXXRW domain and three Asp or Asn residues in the catalytic activity of Chs. By use of hydrophobic cluster analysis (HCA) of different β-glycosyl-transferases, including *S. cerevisiae* Chs1, two conserved domains, one located at the N-terminal half (A), and the other one (B) at the C-terminal half were tentatively identified as important in catalysis. In turn, three aspartic acid residues, two at domain A, and a third one at domain B, plus the motif QXXRW were suggested to be involved in enzyme activity (Saxena 1995). These results were extended later on to additional β-glycosyl-transferases (Campbell et al. 1997), and data of crystallographic analysis of a β-glycosyl-transferase from *Bacillus subtilis* evidenced that the three aspartic residues were involved in binding of the UDP-sugar together with a divalent cation at the active site (Charnock and Davies 1999). Directed mutagenesis of these residues has reinforced these conclusions. Mutation of *S. cerevisiae* Chs2 residues Asp441, Asp562, Gln601, Arg604, and Trp605, the last three belonging to the QRRRW motif led to enzyme inactivation (Nagahashi et al. 1995). In turn, our data of Chs comparison

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demonstrate that the residues Asp441 (or Asn in some Chs), Asp 562 that belongs to the EDRXL motif (Table 3), and QRW, belonging to the QRRRW motif (Table 3), are conserved in all chitin synthases. The third Asp residue recognized in other β-glycosyl-transferases may correspond to one located at position 745 in division 1 enzymes and *Saprolegnia monoica*, probably equivalent to the one located three aa residues beyond in division 2 enzymes, and four aa residues before in Chs from animals (Ruiz-Herrera et al. 2002).

The program QSLAVE, an expert system of protein-fold prediction (Johnson et al. 1993) has been used to solve the structure of the active site of chitin synthase. The consensus from several Chs compared with a large number of different fold families revealed that the results fitted better with a $(\beta/\alpha)_8$-barrel fold family (Horsch and Sowdhamini 1996). Use of the QSLAVE program using human aldolase as template and comparison with a fragment from *Rhizopus oligosporus* Chs1, suggested that the catalytic center of Chs was made by residues Thr88, Tyr90, Asn92, Lys219, and Asp256. However these aa are conserved only in Chs from division 1 and *Saprolegnia*, but not in fungal division 2 or animal enzymes. In contrast, aa from another important cluster: Glu400, Asp401, Leu404, Gln440, Arg443, and Try444, corresponding to aa from the EDRXL and QXRRW motifs (Table 3) are conserved in all Chs. Different results were obtained by analysis using as template a T4 DNA-modifying β-glucosyl-transferase, whose crystal structure in the presence and absence of substrate has been analyzed (Vrielink et al. 1994). The structure of this enzyme reveals a deep central cleft separating a fold similar to the Rossman fold. It is suggested that such cleft constitutes the substrate binding site. The comparison revealed that the following aa residues made direct contact with the substrate: Asn215 (or Asp), Tyr 261, Arg269 (or Lys), and Glu272 (or Gln). Trp341, corresponding to the QRRRW motif also was considered to be important in the hinge region between the two subdomains of the fold (Merz et al. 1999). As seen in Table 3, all of these aa residues are conserved in the different Chs.

We made use of the Psi-Pred program (Jones 1999) to analyze the secondary structure of representatives of the five classes of Chs (Ruiz-Herrera et al. 2002). In analogy to the results obtained by the use of QSLAVE program described above (Horsch and Sowdhamini 1996) we observed that all Chs classes displayed a protein fold of the alternating β/α type. More remarkable is the fact that the relative location of the conserved motifs QXXEY, LP(G/A), LXEDRXL and QXRRW, as well amino acids G629, K662, E665, G669, L714, L720, T744 and P747 (related to Chs1 from yeast) were rather invariant in the structure of the enzymes. Thus, the QXXEY motif appeared at the second helix of the fragments, the LP(G/A) tripeptide appeared in the fifth coil, the LXEDRXL motif was located at the start of the sixth helix, and the QXRRW was surrounded by two coil regions at the end of the seventh helix (Ruiz-Herrera et al. 2002). Globally the results suggest that the peptide secondary structure and the relative location of certain key aa residues responsible for the preservation of catalytic activity in Chs have been conserved during evolution.

9 CONCLUSIONS

Chitin, the most abundant nitrogenous compound in nature, is amply distributed in the eukaryotic kingdoms. In fungi, chitin is responsible for the mechanical resistance of the cell wall. Study of chitin synthesis is important from both a basic and applied points of view. The latter since it represents the ideal target for antimycotics. Additionally, the biosynthetic enzyme chitin synthase (Chs) may be subject to genetic enginrering for increased production of the polysaccharide as substitute for nondegradable plastics in a number of applications. Despite of our increased knowledge on Chs, its mechanism of action remains elusive, and the possibility that it involves lipid or protein acceptors is not a settled issue yet. Chs is synthesized in the ER and mobilized in the cell through the normal exocytic route. Post-golgi specialized microvesicles known as chitosomes, target the enzyme to the sites at the surface where the polysaccharide is synthesized. Newly synthesized chitin chains may be subjected to chemical modification, and/or associated to form microfibrils, and engage into covalent and noncovalent bonds with other wall compounds to constitute a stable composite.

Interestingly, a better knowledge exists of the coding genes (*CHS*) than of the enzymes themselves. A general feature of fungi is to contain more than one Chs. Studies with mutants affected in one, or even two *CHS* genes suggest that their redundancy is in general a protection mechanism that has been selected during evolution. Different chitin synthases are regulated at the transcriptional and post-transcriptional levels by different effectors. Regulatory regions of *CHS* genes from the same organism may contain different regulatory motifs, and share other ones. This feature suggests that they may be under specific and general regulation mechanisms, depending on the environmental conditions. Analysis of the aa sequence of a large number of Chs reveals that they are grouped into two divisions and five classes represented, with a single exception, in all fungal major taxa. Accordingly it appears that division of Chs groups started soon after fungi separated during evolution from the rest of the eukaryotic kingdoms. It has not been possible to perform crystallographic studies on Chs structure. Nevertheless different studies have revealed that Chs belong to the $(\beta/\alpha)_8$-barrel fold family of proteins, and conserve the essential features of β-glycosyl transferases, i.e. the QXXRW motif and three aspartic or asparagine residues at the catalytic site. Chs belonging to all five classes conserve the spatial organization of several common conserved motifs, and invariant aa residues in the form of a protein fold of the alternating β/α type. Taking into consideration the increased interest on chitin synthesis, we may be confident that in a near future we

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will have a complete understanding of Chs structure, as well as of the mechanism of chitin synthesis *in vitro* and *in vivo*.

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Bioactive Fungal Polysaccharides and Polysaccharopeptides

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1 INTRODUCTION

All polysaccharides contain either a pyranose or a furanose carbohydrate ring structure. This common building block can, however, exhibit a wide range of structural properties and functional characteristics. Polysaccharides can be divided into several general categories. Structural polysaccharides include pectins, cellulose, xylan, and mannans. Storage polysaccharides include starch, glycogen, and the fructans—inulin and levan. The marine polysaccharides include alginate, carragenan, agar, agarose, chitosan, and chitosan derivatives. Bacterial polysaccharides include bacterial alginate, xanthan, bacterial dextran and fungal polysaccharides include pullulan, scleroglucan, and schizophyllan. Synthetic polysaccharides include cyclodextrins (Tombs and Harding 1998).

Nonfungal polysaccharides are already being used in numerous commercial applications. The biological activities of many fungal polysaccharides including antitumor, immunomodulatory, hypoglycemic, and hypolipidemic activities have been described. In fact, many mushroom extracts enriched in polysaccharides and polysaccharopeptides (PSPs) are available over the counter as health supplements in pharmacies in the Orient. This chapter illustrates that fungi, especially mushrooms, are a rich source of polysaccharides and PSPs with important biological activities including hypoglycemic, hypolipemic, anti-tumor and immunomodulatory, free-radical scavenging, and antiviral activities.

2 ISOLATION AND CHARACTERIZATION

Various methods have been used for the isolation of fungal polysaccharides and polysaccharide–peptide complexes. Repeated extraction with hot water and cold NaOH has been used for isolating polysaccharides from *Agaricus blazei* and *Sparassis crispa* (Ohno et al. 2000; 2001) followed by fractionation by ion-exchange chromatography on DEAE-Sephadex A-25. Other investigators have used similar protocols involving hot water extraction, gel filtration, and ion-exchange chromatography for isolating polysaccharides from *Sarcodon aspratus* (Mizuno et al. 2000), *Agaricus blazei* (Mizuno et al. 1999), *Omphalia lapidescens* (Ohno et al. 1992; Saito et al. 1992) and *Phellinus linteus* (Song et al. 1995). An additional affinity chromatography step has been used by Mizuno et al. (1995) to isolate polysaccharides from *Tricholoma giganteum*, and by Zhang et al. (1994a) to isolate polysaccharides from *Ganoderma tsugae*. Chromatofocusing purification steps have been used to obtain a proteoglycan from *Agaricus blazei* (Fujimiya et al. 1998). Polysaccharides have been isolated from *Hehenbuehelia serotina* (Ma et al. 1991) by a procedure where the initial extraction is followed by ethanol extraction and then ion-exchange chromatography, gel filtration, and affinity chromatography.

A number of different techniques have been used to characterize isolated polysaccharides and polysaccharide–peptides. Classical structural analysis and heteronuclear multiple quantum coherence nuclear magnetic resonance experiments have been used to investigate the structure of *Boletus erythropus* D-glucan (Chauveau et al. 1996). Methylation analysis, partial acid hydrolysis, and mass spectrometry have been used to determine the structure of *Omphalia lapidescens* glycan (Saito et al. 1992). Partial acid hydrolysis, acetolysis, and chemical analysis of the complete hydrolysates of the fully methylated polysaccharide have been utilized to study the structure of *Flammulina velutipes* polysaccharide (Ikekawa et al. 1982).

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3 ANTINEOPLASTIC AND IMMUNOSTIMULATING ACTIVITIES

The literature pertaining to the anti-neoplastic and immunostimulating activities of fungal polysaccharides and PSPs is voluminous. Different groups of investigators have worked on *Agaricus bisporus*. Polysaccharide–protein complex (antitumor organic substance Mie, ATOM) prepared from cultured mycelia of *Agaricus blazei* by Ito et al. (1997) shows antitumor effects on mice with sarcoma 180 cells, Ehrlich ascites carcinoma, shionogi carcinoma 42 and meth A fibrosarcoma. The effective dosages range from 10 mg/kg/day for 10 days to 100 mg/kg/day for 10 days. The antitumor activity includes activation of macrophages and alterations to the third component of complement but there is no direct cytotoxicity on tumor cells.

A neutral β-glucan has been isolated from cultured fruiting bodies of *Agaricus blazei* by three similar methods that involve repeated extraction with hot water, cold NaOH, or hot NaOH (Ohno et al. 2001). The chromatographic behavior of the glucan on DEAE-Sephadex, its resistance to 1,3-β-glucanase, and the presence of a signal attributable to 1,3-β-glucosidic linkage in ^{13}C-nuclear magnetic resonance spectrum, all suggest that the glucan is a highly branched 1,3-β-glucan.

A glucomannan with a main chain of β-1,2-linked D-mannopyranosyl residues and a side chain of β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl residues has been obtained from the hot water-soluble fraction of mycelia of *Agaricus blazei*, and this shows antitumor activity against sarcoma 180. This molecule does not cross-react with antibodies against antitumor polysaccharides from other mushrooms, or antibodies against a previously isolated polysaccharide from the same mushroom (Mizuno et al. 1999). An α-1,4-glucan-β-1,6-glucan complex from *Agaricus blazei* with a molecular mass of 20 kDa has shown tumoricidal activity and immunostimulation. This compound has *in vitro* cytotoxicity toward Meth A tumor cells but not normal cells. The serum level of immunosuppressive acidic protein is increased, indicating increased migration of granulocytes (Fujimiya et al. 1998). A linear (1 → 3)-αD-glucan, with a molecular mass of 42 kDa, has been isolated from the alkaline extract of *Amanita muscaria* fruiting bodies. This compound does not show antitumor activity against sarcoma S180 in mice, however, its carboxymethylated derivative shows strong activity (Kiho et al. 1994). Similarly the carboxymethylated derivatives of linear (1 → 3)-alpha-D-glucans from fruiting bodies of *Agrocybe cylindracea* and *Amanita muscaria* show higher immunostimulation on murine peritoneal macrophages than the corresponding beta-D-glucans. However, they do not differ in respect of their ability to activate the reticuloendothelial system (Yoshida et al. 1996).

Two antitumor polysaccharides, designated AT-HW and AT-AL, that have been isolated from the hot water and alkaline extracts, respectively, from the fruiting bodies of *Armillariella tabescens*, have been found to suppress the growth of murine sarcoma 180 tumor. These polysaccharides also stimulate the reticuloendothelial system, augment the number of peritoneal exudate cells, activate macrophages, and enhance the mitogenic reaction. AT-HW has a molecular mass of 105 kDa and comprises of mainly beta-(1 → 3)-linked D-glucopyranosyl residues. AT-AL has a molecular mass of 93 kDa and primarily comprises of alpha-(1 → 3)-linked D-glucopyranosyl residues (Kiho et al. 1992).

A water-insoluble glucan has been purified from the fruiting bodies of *Auricularia* species. It has a molecular mass of 610 kDa and has potent antitumor activity against the solid form of sarcoma 180. A further water-soluble, branched (1 → 3)-β-D-glucan, designated glucan I, has been isolated from fruiting bodies of *Auricularia auricula-judae*, and this also shows strong antitumor activity against sarcoma 180 solid tumor implanted in mice. An alkali-insoluble, branched (1 → 3)-β-D-glucan designated glucan II from the same fungus does not have antitumor activity as a purified compound, but acquires such activity after modification by controlled periodate oxidation, borohydride reduction, and mild acid hydrolysis (Misaki et al. 1981).

Domer et al. (1988) have reviewed the immunomodulatory activities of mannan and cell wall-derived glycoproteins from the opportunistic pathogen *Candida albicans*. The mannan extracted with hot citrate buffer is found to be heterogeneous by an antibody forming cell assay. The antibody response is either stimulated or inhibited when mannan is applied together with type III pneumococcal polysaccharide (a T helper cell-independent antigen). or with sheep red blood cells (a T helper cell-dependent antigen). Two cell-wall glycoproteins are found to be responsible for the immunoenhancing activity. Although the mechanism of immunomodulation is not fully known, it is not likely to be attributed to a direct mitogenic effect on lymphocytes or to any enhancement of generation of B cell growth factors or interleukin 2.

A protein-bound polysaccharide from the culture filtrate of *Cordyceps ophioglossoides*, with glucose being the neutral sugar component and galactosamine as the aminosugar, exhibits antitumor activity against syngeneic tumors (Ohmori et al. 1988). Sakagami et al. (1991) have reviewed the immuno-stimulatory activity of PSK, a protein-bound polysaccharide isolated from mycelia of *Coriolus versicolor*. The high molecular mass fraction derived from PSK shows significant antimicrobial activity, enhances the incorporation of radioactive iodine into an acid-soluble fraction of human peripheral blood polymorphonuclear cells, and synergistically augments both tumor necrosis factor (TNF)-induced cytotoxicity against L-929 cells, and the differentiation of human myelogenous leukemia cell lines toward monocytes/macrophages. The PSK fraction also decreases the down-regulation of specific ^{125}I-TNF or ^{125}I-interferon gamma binding to cellular receptors. The PSK provides some protection against carcinogenesis induced by chemical carcinogens and radiation, and prevents pulmonary metastasis of methylcholanthrene-induced sarcomas, human prostate

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cancer and lymphatic metastasis of mouse leukemia, rat hepatoma, and mouse colon cancer. The anti-metastatic action of PSK occurs due to inhibition of: (a) intravasation through the suppression of tumor invasion, adhesion, and the production of cell matrix-degrading enzymes, (b) tumor cell attachment to endothelial cells through suppression of tumor cell-induced platelet aggregation, (c) tumor cell migration after extravasation through repression of tumor cell motility, and (d) tumor growth after extravasation through repression of angiogenesis, modulating cytokine production, and by increasing effector cell functions (Kobayashi et al. 1995). Kato et al. (1983) reported that PSK activates the complement system and raises serum complement level. The highest molecular mass (>200 kDa) fraction of PSK gives the best stimulation of the production of differentiation-inducing factor and cytotoxic factor by the murine macrophage-like cell line J774.1 and interleukin-1-like factor by human peripheral blood monocytes (Kurakata et al. 1991). Hayashida et al. (1991) have found that PSK enhances interleukin-2 induced production of interferon-gamma and TNF-alpha and that combined treatment with cord lymphokine-activated killer cells and PSK can improve advanced retinoblastoma in the neonatal period.

Polysaccharopeptide has been isolated from *C. veriscolor*. A considerable amount of both basic and clinical research has been undertaken with PSP and the literature on this has been reviewed recently (Ng 1998). The peptide–polysaccharide complex activates both macrophages and lymphocytes and shows no direct cytotoxicity against L929 (mouse fibroblast cell line), H3B (mouse hepatoma), and JAR (human placental choriocarcinoma) cell lines (Liu et al. 1993). The molecular mass of PSP is 100 kDa and its polypeptide moiety is rich in aspartic and glutamic acids. The polysaccharide moiety of PSP is made up of monosaccharides with α-1, 4 and β-1, 4 glucosidic bonds. The PSP shows a range of pharmacological activities including hepatoprotective, anti-viral, analgesic, anti-proliferative, antitumor, and immunostimulating activities. In addition, PSP inhibits paracetamol-induced elevation in plasma aminotransferases (Yang 1999). The PSP stimulates the proliferation of human T cells, the function of mouse B cells, cytokine production by macrophages, and natural killer activity in patients undergoing chemotherapy. The PSP also shows immunomodulating effects on lymphokine-activated killer cells and tumour infiltrating lymphocytes and reduces the dose of interleukin-2 required when co-administered with interleukin-2. It can partly reinstate the immune function suppressed by cyclophosphamide treatment in rats (Yang 1999) and can also inhibit the proliferation of human gastric cancer cell line (7907), human lung cancer cell line (SPC), and human leukemia cell line (MCL) as well as inhibiting tumor growth in mice inoculated with sarcoma 180 cells, sarcoma H238 cells, and human nascopharyngeal carcinoma cells. It has also been reported that PSP significantly enhances the inhibition of ionizing radiation on proliferation of C6 glioma cells (Yang 1999). However, Liu et al. (1993) have not found any cytotoxic effects of PSP on five tumor cells lines including P388D1 (mouse monocyte-macrophage), mouse sarcoma S180, PU5-1.8 (mouse monocyte-macrophage), mouse melanoma B16, and human choriocarcinoma JAR. The discrepancy between this observation and other findings may be due to the cell lines examined. Liu et al. (1993) have also shown that PSP treatment up-regulates the generation of reactive nitrogen intermediates, reactive oxygen intermediates, and tumour necrosis factor. A purified PSP from *C. versicolor* has been shown to inhibit proliferation of a human hepatoma cell line (HepG2). Treatment of human leukemia cells (HL60) with PSP results in inhibition of cell growth, increase in DNA synthesis time, inhibition of cell division through G2/M phase, and an elevation of cyclin B1 expression at the molecular characteristics G2/M phase. A comparison of the immunostimulatory and antitumor activities of *C. versicolor* PSP has been made with polysaccharide–peptides from *Tricholoma mongolicum* and *Tricholoma lobayense*. It has been found that PSP is less potent when compared on a per unit weight basis and more potent when compared on a molar basis. This is due to the much higher molecular mass of PSP. PSP can boost the immune status of gastric, esophageal, lung, and gynecologic cancer patients undergoing radiotherapy or chemotherapy and can mitigate the adverse side effects of radiotherapy and chemotherapy, thereby improving the quality of life.

Polysaccharopeptide does not adversely affect ovarian steroidogenesis, ovulation, gestation, or embryonic development in mice and does not have subchronic toxicity in rats. It does not cause mutagenicity in the Ames test and does not increase either chromosome aberrations in mouse or the number of micronucleated polychromatic erythrocytes (Yang 1999).

A (1 → 6)-branched (1 → 3)-beta-D-glucan of 550 kDa with anti-inflammatory activity in both carrageenan-induced edema and scalded edematous hyperalgesia in rat hindpaws, has been isolated from an alkaline extract of fruiting bodies of *Dictyophora indusiata*. It comprises of a main chain of beta-(1 → 3)-linked D-glucopyranosyl residues with two single, β-(1 → 6)-linked D-glucopyranosyl groups attached as side chains to, on average, every fifth residue of the main chain. The polysaccharide displays antitumor activity against subcutaneously implanted sarcoma 180 in mice (Hara et al. 1983; Ukai et al. 1983). A glucomannan-protein from the cell-wall fraction of *C. albicans* has been found to stimulate production of interleukin-2 and γ-interferon by human peripheral blood mononuclear cells (Ausiello et al. 1987). A β-(1 → 3)-glucan from *Flammulina velutipes* shows antitumor activity. The polysaccharide is composed of D-glucose (42.3%), D-galactose (17.3%), D-mannose (12.2%), D-xylose (6.7%), and L-arabinose (14.7%) and is the most potent antitumor polysaccharide from *F. velutipes* (Ikekawa et al. 1982). A further polysaccharide of 200 kDa from the alkali-soluble fraction of *F. velutipes* (Leung et al. 1997) that has a backbone composed of β-(1 → 3)-D-linked glucose, shows potent antitumor activity against sarcoma SC-180 *in vivo* but not *in vitro*. Proliferation of splenic lymphocytes is observed after intraperitoneal injection of the polysaccharide.

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The protein-bound polysaccharide EA6 from *F. velutipes* fruiting bodies, used together with or after injection of murine leukemia L1210 vaccine, can significantly prolong the life-span of mice challenged with leukemic cells (Otagiri et al. 1983). Two water-soluble polysaccharide–protein complexes with 9.3 and 25.8% protein contents, respectively, have been isolated from the mycelium of *Ganoderma tsugae*. These have shown antitumor activity against sarcoma 180 in mice (Zhang et al. 1994a) and a further antitumor polysaccharide has been isolated from *Ganoderma lucidum* (Miyazaki and Nishijima 1981). Both the insoluble and soluble high-molecular-weight forms of grifolan, a 1,3-β-glucan from *Grifola frondosa*, can stimulate macrophages to produce TNF *in vitro*. This polysaccharide has shown both antitumor and immunomodulating activities. It stimulates the alternative complement pathway, glucose consumption by macrophages, and macrophage-mediated lysosomal enzyme activity. Heat treatment of the glucan generates fragments with reduced biological activities. Besides antitumor effects, the glucan also shows an immunoenhancing activity (Ishibashi et al. 2001). Other polysaccharides with antitumor activity have been isolated from fruiting bodies of *Hericium erinaceum* (Mizuno et al. 1992). A mixture comprising of glucose-containing heteroglycan, β-D-glucan with galactose-containing heteroglycan and protein, and a (1 → 6)-β-D-glucosyl branched (1 → 3)-β-D-glucan, has been isolated from fruiting bodies of *Hohenbuehelia serotina*. This mixture has antitumor activity against mice bearing sarcoma 180 (Ma et al. 1991).

Lentinan is a polysaccharide with marked antitumor activity produced by *Lentinus edodes*. Lentinan stimulates lymphokine-activated natural killer cell activity and cytotoxic macrophages. Lentinan is more effective in inhibiting spontaneous pulmonary metastasis when used in combination with interleukin-2 (Yamasaki et al. 1989; Tani et al. 1992). A xylose-rich heteroglycan-protein fraction (LAP1), prepared from a solid culture of actively growing *L. edodes* mycelia (Hibino et al. 1994), stimulates production of nitrite and interferon-γ in murine splenocytes. A highly branched (1 → 3)-beta-D-glucan from *Omphalia lapidescens* has been shown to have antitumor activity against the ascitic forms of sarcoma 180 and MH-134 when co-administered with 5-fluorouracil but is ineffective against the solid forms of sarcoma 180 (Ohno et al. 1992). A polysaccharide–protein complex of 153 kDa, isolated from a hot water extract of cultured mycelia of *Phellinus linteus*, stimulates β-lymphocytes to produce polyclonal antibody. The complex consists of 82.5% polysaccharide and 13.2% peptide and the polysaccharide moiety is made up of arabinose, galactose, glucose, mannose, uronic acid, and mannose, with mannose being the predominant species. The protein moiety is made up of 10 amino acids with aspartic and glutamic acids being the major components (Song et al. 1995).

A number of polysaccharide–protein complexes from *Pleurotus citrinopileatus* have been shown to have activity against sarcoma 180 implanted in mice. They include: (a) a heteropolysaccharide made up of glucose, mannose, arabinose, and galactose with about 10% protein, (b) a complex made up of 60% protein and 40% glycan, which in turn is composed of fucose, galactose, glucose, mannose, and xylose, (c) a complex made up of 50% protein and 50% glycan, which consists of the same types of sugar as those possessed by (b), (d) a complex of 680 kDa and made up of 80% (1 → 3)-β-D-glucan and 20% protein, (e) a complex made of 68% (1 → 3)-β-D-glucan and 32% protein, (f) a complex of 1900 kDa and made up of 8.7% β-D-glucan and 13% protein, and (g) a complex of 1200 kDa and made up of 87% β-D-glucan and 13% protein (Zhang et al. 1994b). A β-(1 → 3)-linked glucan with branches of galactose and mannose residues, isolated from a hot water extract of the mushroom *Pleurotus ostreatus*, has shown pronounced antitumor activity at 0.1 mg/kg body weight. A further two polysacchardies that do not show antitumor activity have also been isolated from the same fraction. One of these is also a β-(1 → 3)-linked glucan with a higher proportion of acidic sugars than the antitumor polysaccharide and the other polysaccharide is composed of α-linked galactose and mannose residues (Yoshioka et al. 1985).

A 5000 kDa (1,3)-(1,6)-beta-D-glucan from the mycelia of *Poria cocos* shows antitumor activity (Kanayama et al. 1983). An heptoglucoside isolated from *Saccharomyces cerevisiae* cell walls is a unit ligand for human monocyte beta-glucan receptors (Janusz et al. 1986). Zymosan contains beta-(1 → 3)-D-glucan and α-mannan in the ratio 1:1 and shows antitumor activity in sarcoma-bearing mice (Whistler et al. 1976). Opsonized zymosan particles stimulate platelet activating factor synthesis by monocytes (Sakurai et al. 1992). D-mannan and D-glucan from yeast have shown antitumor activity in mice (Suzuki et al. 1969). A fucogalactan, isolated from the hot water extract of *Sarcodon aspratus*, causes the release of TNF-α and nitric oxide from murine macrophages (Mizuno et al. 2000). Schizophyllan is a neutral glycan from the culture filtrate of *Schizophyllum commune*, this can augment the production of interferon-γ and interleukin-2 from mitogen-stimulated human peripheral blood mononuclear cells (Sakagami et al. 1988). It may also directly activate peritoneal macrophages, causing them to become cytotoxic (Sugawara et al. 1984). A soluble, highly branched (1 → 3)-beta-D-glucan from *Sclerotinia sclerotiorum* enhances phagocytic activity, lysosomal enzyme activity, active oxygen secretion, and cytokine production (Sakurai et al. 1995). Branched 1,3-β-glucans from cultured fruiting bodies of *Sparassis crispa* demonstrate antitumor activity in mice with the solid form of sarcoma 180 and cause an enhanced hematopoietic response in mice with cyclophosphamide-induced leukopenia (Ohno et al. 2000).

Various heteroglycans with antitumor activity on sarcoma 180 implanted in mice have been obtained from *Tricholoma giganteum* (*Tricholoma lobayense*) (Mizuno et al. 1995). These include (a) a mixture of α-D-glucan and xyloglucomannan with an average molecular mass of 1600 kDa, (b) a 40 kDa β-D-glucan with 1% protein, (c) a 52 kDa (1 → 3)-β-D-glucan with 7.8% protein, (d) three

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(1 → 6)-β-D-glucosyl-branched (1 → 3)-β-D-glucans containing small quantities of xylose and galactose and 3.5–8.3% protein, with molecular mass from 260 to 410 kDa, and (e) a 68 kDa complex of 37.5% protein and 62.5% polysaccharide composed of glucose, mannose, galactose, and xylose.

A peptide-bound polysaccharide of 154 kDa from culture filtrate of *T. lobayense* inhibits the growth of sarcoma 180 cells in mice, restores the phagocytic function of peritoneal exudate cells and mitogenic activity of T cells in tumor-bearing mice, and induces gene expression of some immunomodulatory cytokines. Another peptide-bound polysaccharide of 17 kDa has been obtained from the submerged mycelial culture of *T. lobayense*. The peptide content is only about 8% and it stimulates the proliferation of T cells and nitrite production by peritoneal macrophages. The growth of several tumor cell lines including 388D1 (mouse monocyte-macrophage), mouse sarcoma S180, human hepatoma H3B, and human neuroblastoma SY5Y *in vitro* is unaffected, but antitumor activity against sarcoma 180 has been demonstrated *in vivo*. This polysaccharide shows stronger immunomodulatory and antitumor activities than the protein-bound polysaccharide from *C. versicolor*. Another peptide-bound polysaccharide, 15.5 kDa in molecular mass, has been prepared from the cultured mycelia of *T. mongolicum*. It can activate macrophages, stimulate macrophage antigen-presenting activity, and this, in turn, enhances T-cell proliferation and inhibits growth of sarcoma 180 cells implanted in mice (Wang et al. 1995; 1996).

A (1 → 3)-β-D-glucan branched by O-6 substitution, obtained from the cold-alkali extract of *Volvariella volvacea* fruiting bodies, has shown strong antitumor activity against tumors in mice (Kishida et al. 1989).

4 OTHER MEDICINAL EFFECTS

The bulk of the data on biological activities of fungal polysaccharides and PSPs is on their immunomodulatory and antitumor activities. The other biological activities, which are mentioned below, have been studied only in a few species.

A polysaccharide from cultures of *Cordyceps sinensis* shows hypoglycemic activity in genetically diabetic mice, streptozotocin-induced diabetic mice, and normal mice. However, the plasma insulin level is unaffected although hypocholesterolemic and hypotriglyceridemic effects are observed (Kiho et al. 1988). The polysaccharide is 45 kDa in molecular mass and is composed of galactose, glucose, and mannose in the molar ratio of 62:28:10. Two glycans, ganoderans A and B, isolated from the water extracts of fruiting bodies of *Ganoderma lucidum* and *G. japonicum*, show marked hypoglycemic activity in normal and alloxan-induced diabetic mice (Hikino et al. 1985). A highly branched galactomannan produced by a *Pestalotiopsis* species, shows hypoglycemic activity in streptozotocin-induced diabetic mice and affects oral glucose tolerance in normal mice after intraperitoneal administration. It has a molecular mass of 24 kDa and a galactose:mannose ratio of 1:7. In addition to α-D-mannopyranosyl residues of a yeast mannan type, it also contains β-(1 → 3)-linked D-galactofuranosyl and non-reducing terminal β-D-galactofuranosyl residues (Kiho et al. 1997).

An acidic polysaccharide of 1500 kDa and composed of mannose, xylose, glucuronic acid, and glucose has been isolated from the hot-water extract of the fruiting bodies of *Tremella aurantia*. It shows pronounced hypoglycemic activity in normal mice, streptozotozin-induced mice, and genetically diabetic mice and no harmful effects have been reported (Kiho et al. 1995). A highly branched glucuronoxylomannan from the fruiting bodies of a related species, *Tremella fuciformis*, gives a dose-dependent hypoglycemic action in normal as well as streptozotocin-induced diabetic mice. Plasma insulin level and hepatic activities of hexokinase and glucose-6-phosphate dehydrogenase are elevated while hepatic glucose-6-phosphatase activity is depressed (Kiho et al. 1981). Polysaccharide extracts prepared from fruiting bodies of *G. lucidum*, *Grifolia umbellate*, *T. lobayense*, *V. volvacea*, *T. fuciformis*, and *C. versicolor* have shown free radical scavenging activities (Liu et al. 1997). Polysaccharopeptide inhibits gastric ulcer formation in experimental models including stress restraint, pyloric ligation, acetic acid-induced and pyloric ligation-induced ulcer, but does not affect the amount of gastric juice, active pepsin, free acidity, and PGE_2 content in gastric juice (Hu and Zhou 1999). Polysaccharopeptide from *C. versicolor* inhibits HIV-1 reverse transcriptase and glycophydrolases (Collins and Ng 1997).

5 ROLE IN BIOTECHNOLOGY

Aureobasidium pullulans is used for the industrial production of pullulan. Pullulan is a water-soluble, neutral, linear homopolysaccharide produced from glucose molecules held together by α-1,4 glucosidic bonds to form repeating maltotriose units, which in turn are linked together by α-1,6 glucosidic bonds. Its molecular mass, which ranges from 5 to 10^3 kDa, is dependent on the strain, substrate, pH, phosphate supplements, and the duration of fermentation. Pullulan production can be induced by glucose, fructose, some disaccharides, and nitrogen limitation (Tombs and Harding 1998). Pullulan minimizes sugar crystallization and thus sandiness in ice-cream. It has also been used for formulating snack foods in Japan based on both cod eggs and powdered cheese. Pullulan has low oxygen permeability and cross links with other materials to form thin polymeric films with lower permeability than cellophane and polypropylene. It is used as a packaging film for food such as ham to prevent food oxidation. It also has applications in adhesives, coatings, films, molded articles, and as a flocculating agent in clay suspensions in mining operations. It can be employed as a standard polysaccharide for comparative measurements with other polysaccharides and may also be used to standardize

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instruments (Tombs and Harding 1998; Marwaha and Arora 1999).

Scleroglucan is produced by *Sclerotium glucanicum* and related species while schizophyllan is isolated from *Schizophyllum commune*. These are large, neutral polysaccharides with a molecular mass of around 500 kDa in the form of triple helices stabilized by hydrogen bonds (Yanaki et al. 1980). Both scleroglucan and schizophyllan have immunostimulatory effects. Scleroglucan shows pseudoplasticity over a wide range of pH, temperature, and salt conditions. It is used in cosmetics, in pesticides to enhance binding to leaves, in oil-well drilling fluids due to its thermostability and water-binding capacity, and also in ceramic glazes, printing inks, and latex paints (Tombs and Harding 1998).

Chitosan is the most important solubilized derivative of chitin and is made up of β(1 → 4)-linked *N*-acetyl-D-glucosamine residues. Crustaceans and fungi are the major sources of chitosan. Chitosan can be used in food biotechnology as fining agents for fruit juices and beers, and for the recovery of β-carotene in during processing of carrot juice. Porous bead technology involving chitosan can be used for chelation of heavy metals and affinity chromatography. Chitosan-based gel beads are used for trapping microbes, plant and mammalian cells, and enzymes and the external application of chitosan to crops increases resistance to fungal attack. Chitosan is also used in gene therapy as microadhesives for drug delivery, as bandage contact lenses for corneal healing, in wound healing technology, in the cosmetic industry for hair-setting sprays and cream, nail varnishes and skin creams, shampoos and hair conditioners (Tombs and Harding 1998).

6 CONCLUSION

Many fungal polysaccharides exhibit antitumor and immunomodulatory activities. Fungi produce a diverse range of polysaccharides (Table 1), and these are well known for their immunomodulatory and antitumor activities. They may also exhibit anti-viral, hypoglycemic, hypolipidemic, and free-radical scavenging activities (Table 2). The Japanese preparation of *C. versicolor* protein-bound polysaccharide designated as PSK, the Chinese preparation of *C. versicolor* PSP and polysaccharide preparations from *Ganoderma lucidum* are among the most popular fungal polysaccharides available in pharmacies in the Far East. These polysaccharides have become popular partly due to reports of

Table 1 Structures of some fungal polysaccharides

Fungal species	Structure(s) of polysaccharide(s)
A. blazei	(1 → 3)-α-D-glucan, (1 → 3)-β-D-glucan, α-(1,4)-glucan-β-(1,6)-glucan
A. cylindracea	(1 → 3)-α-D-glucan
A. muscaria	(1 → 3)-α-D-glucan
A. tabescens	(1 → 3)-α-D-glucan, (1 → 3)-β-D-glucan
Auricularia sp.	(1 → 3)-β-D-glucan
C. ophioglossoides	Protein-bound polysaccharide
C. versicolor	Protein-bound polysaccharide, peptide-bound polysaccharide
D. indusiata	(1 → 6) branched (1 → 3)-β-D-glucan
F. velutipes	(1 → 3)-β-D-glucan
G. tsugae	Protein-bound polysaccharide
G. lucidum	Polysaccharide
G. frondosa	(1 → 3)-β-D-glucan
H. serotina	(1 → 6)-β-D-glucosyl branched (1 → 3)-β-D-glucan
L. edodes	Xylose-rich heteroglycan-protein, glycan
O. lapidescens	(1 → 3)-β-D-glucan
Pestalotiopsis sp.	Galactomannan
P. linteus	Peptide-bound polysaccharide
P. citrinopileatus	Protein-bound polysaccharide
P. ostreatus	(1 → 3)-β-D-glucan
P. cocos	(1 → 3)-(1 → 6)-β-D-glucan
S. aspratus	Fucogalactan
S. sclerotiorum	(1 → 3)-β-D-glucan
S. crispa	(1 → 3)-β-D-glucan
T. fuciformis	Glucuronoxylomannan
T. giganteum	Heteroglycan, peptide-bound polysaccharide
T. mongolicum	Peptide-bound polysaccharide
V. volvacea	(1 → 3)-β-D-glucan

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Table 2 Biological activities of some fungal polysaccharides/polysaccharopeptides

Fungal species	Biological activities
C. versicolor	Antitumor, immunomodulatory, free-radical scavenging, antiviral
F. velutipes	Antitumor, immunomodulatory
G. lucidum	Antitumor, hypoglycernic, free-radical scavenging
T. lobayense	Antitumor, immunomodulatory, free-radical scavenging
V. volvacea	Antitumor, free-radical scavenging

immunomodulatory and antitumor activities in cancer patients. They generally improve the immune status and survival rate in cancer patients undergoing radiotherapy or chemotherapy.

Some of the fungi that produce polysaccharides or protein/peptide-bound polysaccharides with immunomodulatory and antitumor activities are easily available from local vendors in the Far East. They include *A. blazei*, *A. cylindracea*, *Auricularia* spp., *Cordyceps* spp., *C. versicolor*, *D. indusiata*, *F. velutipes*, *G. lucidum*, *G. frondosa*, *H. erinaceum*, *L. edodes*, *P. ostreatus*, and *Tremella* spp. *A. cylindracea*, *F. velutipes*, *G. frondosa*, *L. edodes*, and *P. ostreatus* are available as fresh material while the others are commonly available in the dried state. Fungal polysaccharides, PSPs, and protein-bound polysaccharides are extracted with hot water and can thus resist boiling during food preparation and are relatively nontoxic. Some fungal polysaccharides have proven efficacy when administered orally, e.g., PSP from *C. versicolor*.

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Biotechnological Potential of Fungal Lipids

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1 INTRODUCTION

Lipids are very important cell constituents, which can function either as structural components or as carbon and energy reserves. These "basal" molecules are also well known as precursors of compounds, such as prostaglandins or thromboxanes, which play a very important role in the physiology of animal cells. Although mammals use fat as a storage form of energy, they cannot synthesize the very specific lipid molecules which they need, particularly polyunsaturated fatty acids (PUFAs). Since they are unable to introduce double bonds at carbon atoms between C-9 and the terminal methyl group, in order to synthesize ω-3 as well as ω-6 unsaturated fatty acids, such as linoleic (18:2 $\Delta^{9,12}$), α-linolenic (18:3 $\Delta^{9,12,15}$), or γ-linolenic (18:3 $\Delta^{6,9,12}$), they have to obtain these from exogenous producers (i.e., plants or micro-organisms) through their food. The idea of supplementing human food, particularly that of infants, with exogenous PUFAs (Certik and Shimizu 1999) or modifying lipids in order to produce reduced calories structured lipids (Fomuso and Akoh 1997) is widely developed. Most of the oils used in animal nutrition are produced by plants but, if these sources do not contain enough of certain essential fatty acids, it is often necessary to modify their fatty acid content (Gunstone 2001). The production of novel plant oils (Murphy 1999) and possible alternative sources Gunstone (1999) have been thoroughly reviewed. Micro-organisms (Certik and Shimizu 1999) are able to accumulate fats (Meesters et al. 1996; Ratledge and Wynn 2000). Among them, fungi, which are metabolically very versatile micro-organisms, many of which have been shown to be safe for the production of food ingredients (Streekstra 1997) can be used on several ways:

- Direct extraction of high value fat accumulated in the cells, using efficient procedures (Chen and Chi 1994; Certik et al. 1996; Somashekar et al. 2001), which do not induce any alteration of fatty acids (Walker et al. 1999; Certik and Horenitzky 1999).
- Transformation of exogenous fat by growing an appropriate strain in a fermentor or bioreactor (Konova and Pankina 1997; Kinoshita and Ota 2001; Pelesane et al. 2001).
- Production of specific lipases for the transformation of exogenous lipids in a bioreactor (Huang et al. 1994; 1997; Jennings and Akoh 1999; 2001).
- As donors or hosts for specific genes that can be expressed in "oleaginous" fungi (Budziszewski et al. 1996; Sakuradani et al. 1999a,b; Certik and Shimizu 1999).

This chapter will focus on the production of PUFAs, surfactants, and lipases by selected fungal strains. The use of fungal lipases in the modification of common oils (especially for enrichment with PUFAs) will also be reviewed, as well as the application of molecular methods (such as gene transfers), which can lead to the combination of abilities to produce substantial amounts of specific lipids, e.g., with suitable PUFAs composition or having surfactant properties.

Since two book chapters on the biotechnology of fungal lipids (Weete and Gandhi 1992; Sancholle and Lösel 1995) have already provided a good overview of this topic and contain the major data prior to 1994, the present review will mainly deal with subsequent developments.

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2 PRODUCTION OF POLYUNSATURATED FATTY ACIDS (PUFAs)

The total lipid content of fungi may vary from less than 1% to about 87% of dry weight (Weete 1980). Several genera of yeast and filamentous fungi, called "oleaginous fungi," may produce from 48 to 66% of their dry weight as lipid (Weete and Gandhi 1992), using waste substrates (Cheng et al. 1999), including whey (Abou 1994; El Kady et al. 1995), fat from wastewater (Funtikova et al. 1999a), chloroanilines (Funtikova et al. 1999b), or apple pomace (Stredanski et al. 2000). Polyunsaturated fatty acids are easily available only if they occur in the triacylglycerols fraction. Within the same species, lipid content and composition may vary greatly with strains (Jansen van Rensburg et al. 1995), culture time (Wynn et al. 2001), and phases of the life cycle (Jansen van Vuuren et al. 1994; Lodewyk et al. 1993). Such changes may be monitored by mathematical models, which may help in predicting the accumulation of lipids as well as the fatty acid content (Aggelis et al. 1995a,b) and in avoiding their degradation in these oleaginous fungi (Aggelis and Sourdis 1997).

The "ancestral" Protoctistan fungi are characterized by the presence of the ω-6 series of C18 and C20 PUFAs, the Zygomycetous fungi by the presence of mainly ω-6 C18 PUFAs and the Dicaryomycota by the presence of ω-3 and not ω-6 PUFAs (Vanderwesthuizen et al. 1994). The fatty acid composition of fungi has recently been reviewed by Kock and Botha (1998) who discussed the use of fatty acid profiles in the identification of yeasts (Botha and Kock 1993), particularly yeasts which are agents of oral diseases (Blignault et al. 1996). Oleaginous fungi produce large amounts of fat but they are of relatively low interest if their fatty acid composition is of a common type, as in *Aspergillus sydowii*, *Fusarium oxysporum*, or *F. equiseti*, in which the major fatty acid is oleic (Azeem et al. 1999). On the contrary, the fungal strains, which synthesize large amounts of lipid containing rare PUFAs like ω-6 C18:3, 20:4, 20:5 or ω-3 C22:6 are valuable (Ratledge and Wynn 2000), provided these PUFAs are present in the readily extractable triacylglycerols fraction. Since exogenous stress factors may change the amount and composition of fungal lipid (Zalashko et al. 2000), there are possibilities for influencing fungal lipid synthesis *in vitro* by means of the appropriate stress. The γ-linolenic acid content may be strongly influenced by culture conditions or ageing of the mycelium (Xian et al. 2001). Particular attention should be paid to the elegant review of Certik and Shimizu (1999) who mention γ-linolenic acid (GLA), di-homo-γ-linolenic acid (DHGLA), arachidonic acid (AA), eicosatetraenoic acid (3 HETE), eicosapentaenoic acid (EPA), and docosohexaenoic acid (DHA) as the most important PUFAs which can be obtained from fungi.

2.1 γ-Linolenic Acid

This ω-6 polyunsaturated fatty acid is a precursor of the prostaglandins of the PGE_1 series. It was recently shown to exert a beneficial influence on the deformability of red blood cells in hemodialysis patients (Shigeruko et al. 2000). Dietary mold oil rich in γ-linolenic acid, relative to linoleic acid, increases glucose metabolism in response to insulin stimuli in isolated rat adipocytes (Takashi et al. 2001). Studying the effect of γ-linolenic acid on the growth and metastasis of a human breast cancer, Rose et al. (1995) showed that this fatty acid is metabolized *in vivo* to arachidonate-derived eicosanoids, which are involved in the metastatic process. However, while linoleic acid stimulated tumor cell invasion *in vitro* and 92-kDa type IV collagenase production, GLA inhibited invasion and did not induce the activity of the proteolytic enzyme.

2.1.1 Producers

Even if some Dikaryomycota were shown to contain GLA (Pohl et al. 1997), the best producers belong to the Mucorales (Zygomycotina), two genera of which were used for industrial production: *Mucor javanicus* in the United Kingdom and *Mortierella isabellina* in Japan (Gunstone 1999). The GLA represents more than 15% of the lipid of *Absidia corymbifera*, *Mucor rouxii*, *Mucor genevensis*, *Mucor ambiguus*, *Conidiobolus coronatus*, and *Cunninghamella echinulata*, which produced as much as $577\,mg\,L^{-1}$ GLA (Chen and Chi 1994). Funtikova et al. (1998) screened 22 species of the genera *Mucor* and *Rhizopus* and found that *Mucor circinelloides* may produce 20–31 mg of GLA per g of absolutely dry biomass.

2.1.2 Influence of Carbon Sources

Very good production ($697\,mg\,L^{-1}$) of GLA was obtained with batch cultures of *Mortierella ramanniana* (Leman 1997) but, with 25% w/v of glucose as carbon source (C/N = 19), too expensive for industrial production. Hiruta et al. (1996a) reported that a low temperature-resistant mutant MM15 of *M. ramanniana* grown in a 600-l fermentor may produce lipid with 18.3% GLA. The scale-up to a 10-kL fermentor with glucose ($300\,g\,L^{-1}$) as carbon source confirmed these results and showed that the pellet form obtained in fermentors is better for GLA production than the filamentous one (Hiruta et al. 1996b). Several species from the genus *Mucor* are better producers than some *Mortierella*, e.g. *Mucor circinelloides* (36.4%) vs. *Mortierella alpina* (5.61%) (Botha et al. 1997). Moreover, *M. circinelloides* may use cheaper carbon sources, like short-chain monocarboxylic acids (du Preez et al. 1996). When acetic acid, a good substrate for this species, is used as carbon source, the maximum GLA content comes after nitrogen exhaustion and may reach 39.8% of dry weight, corresponding to a production of $350\,mg\,L^{-1}$ (Du Preez et al. 1995), with an acetic acid concentration as low as $2\,g\,L^{-1}$ (du Preez et al. 1997). With a C/N ratio of 25:1, GLA production reached $510\,mg\,L^{-1}$ culture medium, but decreased at lower ratios (Immelman et al. 1997). High C/N ratios are essential because accumulation of cellular lipids occurs only after nitrogen exhaustion (Kavadia et al. 2001). The effects of

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nitrogen sources on the activities of lipogenic enzymes of fungi have also been studied (Certik et al. 1999). Funtikova and Mysyakina (1997) showed that fatty acids are not very good carbon sources for GLA production. However, the utilization of oils like sunflower oil may be improved by adding acetate to the culture medium (Jeffery et al. 1997; 1999). This confirms the statement of Kendrick and Ratledge (1996) about the cessation of production of PUFAs, when some fungi are grown on plant oils. A good cheap carbon source for GLA production may be fat present in wastewater, the GLA content depending on the amount of lipid contaminants present (Funtikova et al. 1999a). When the culture medium contains ethanol, desaturation reactions are favored and γ-linolenic acid oxidation in the late stationary phase is inhibited (Emel'yanova and Eroshin 1996).

2.1.3 Culture Conditions

Other cultural factors may influence the production of γ-linolenic acid: dissolved oxygen tension (Roux et al. 1995), incubation time, growth temperature (Carvalho et al. 1999), form, and density of inoculum (du Preez et al. 1995; Chen and Liu 1997). A shake culture of *Cunninghamella echinulata* produced up to 1,35 mg L^{-1} γ-linolenic acid (Chen and Liu 1997). Using *Mortierella ramanniana* in a Maxblend fermentor, Hiruta et al. (1997) obtained a very significant increase of GLA production.

2.1.4 Solid-State Fermentation

Solid-state fermentation looks promising for GLA production using cheap substrates or wastes. Among several Mucorales strains grown on cereals by Conti et al. (2001), *Cunninghamella elegans* gave the best results, producing 14.2 mg of GLA per gram of dry substrate after 11 days of cultivation. *Thammidium elegans* was also successfully grown on apple pomace for production of γ-linolenic acid (Stredanski et al. 2000).

2.2 Eicosapolyenoic Acids

All of the C_{20} PUFAs, such as dihomo-γ-linolenic acid (DHGLA, 20:3n-6), arachidonic acid (AA, 20:4n-6), and 5,8,11,14-17-cis-eicosapentaenoic acid (EPA, 20:5n-3) are precursors for prostaglandins, thromboxanes, leukotrienes, or prostacyclins, which have hormone-like characteristics. Since these PUFAs exhibit several unique biological activities, such as lowering of plasma cholesterol level or prevention of thrombosis (Ogawa et al. 2002), an intensive search for industrial producers of these valuable fatty acids is in progress. Most of them accumulate simultaneously in the same organism. Only Δ^5 desaturase-defective mutants of *Mortierella alpina* do not produce arachidonic acid together with DHGLA (Kawashima et al. 2000). Following the study by Certik and Shimizu (1999), a beautiful biosynthetic pathway explaining this simultaneous production was proposed by Ogawa et al. (2002).

2.2.1 Producing Organisms

If members of the genus *Mucor* are the best producers of GLA, *Mortierella* species are by far the best for the C20 series of PUFAs, particularly arachidonic acid (Aki et al. 2001; Botha et al. 1999; Eroshin et al. 2000; Pan'kina and Konova 1998; Sajbidor et al. 1994; Streekstra 1997). An almost exhaustive screening of *Mortierella* species, with regard to their AA production was made by Eroshin et al. (1996). *M. alpina* is widely used (Eroshin et al. 2000; Higashiyama et al. 1998a,b; Kawashima et al. 1997; 1998), as well as *M. elongata* (Cheng et al. 1999) or *M. alliacea*, which is able to produce up to 5 g L^{-1} AA (Aki et al. 2001). Selected Oomycetes are good producers of EPA, accompanied by AA. The isolate MA-2801 of *Achlya* sp produces simultaneously EPA and AA in a good ratio (Aki et al. 1998). *Pythium debaryanum* may yield up to 135 mg L^{-1} C20:5 acid plus 197 mg L^{-1} C-20:4, after 172 h growth (Soloveva et al. 1997), while species from the genus *Saprolegnia* produce AA and EPA in amounts exceeding 30% of the total FA content, with an AA/EPA ratio ranging from 1.08 to 3.24 (Galanina and Konova 1999). *Pythium irregulare* also produces reasonable ratios of EPA and AA (Cheng et al. 1999).

2.2.2 Culture Conditions

The production of EPA and AA my be influenced by cultural conditions, inducing a shift towards the preferential production of one of these eicosapolyenoic fatty acids. Such a shift may occur spontaneously in the course of fungal growth (Galanina et al. 1999). The use of starch as main carbon source in pre-pilot scale cultivation improved the production of AA by *Mortierella alliacea* to 5.0 g L^{-1} on day 5 (Aki et al. 2001). The AA production by *M. alpina 1S4* may be enhanced 1.7-fold by mineral addition (Higashiyama et al. 1998a) and may reach 10.9 g L^{-1} after 8 days of culture in a 10-kl industrial fermentor (Higashiyama et al. 1998b). The nitrogen source may also influence production of AA by *M. alpina*, potassium nitrate being better than urea (Eroshin et al. 2000). When *Pythium irregulare* is grown on 4% crude soybean oil with 0.2% Tween, the EPA production is 700 mg L^{-1} but may be doubled by adding only 1% soymeal waste to the substrate (Cheng et al. 1999). Temperature may also influence production qualitatively, EPA and AA being respectively predominant at 12 or 18°C. By combining the effects of substrate supplementation and temperature, the AA/EPA ratios may range from 0.2 to 4.0, which would be reasonable for food additive or supplement applications, e.g., infant formula (Cheng et al. 1999). A good source of nitrogen for the production of EPA and AA by *Pythium debaryanum* is protein hydrolysate obtained from food industry waste (Soloveva et al. 1997). These culture conditions may result in more or less good bioavailability of AA as a function of its

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association with other fatty acids in the triacylglycerol molecule (Liu et al. 1998).

2.2.3 Production of Dihomo-γ-Linolenic Acid (DHGLA)

Always present in small amounts in cultures of fungi producing AA and EPA, DHGLA is synthesized by elongation of GLA; DHGLA is the precursor of AA, which results from it by Δ^5 desaturation, according to the biosynthetic pathway described by Certik and Shimizu (1999). Strains with moderate AA content may produce higher amounts of DHGLA and GLA when they are grown on a medium containing acetylsalicylic acid, which blocks Δ^5 desaturation (Eroshin et al. 1996). It is thus possible to improve DHGLA concentration by using inhibitors of Δ^5 desaturation. Addition of 3% sesame oil to a glucose-yeast extract medium increased the production by *M. alpina 1S-4* to a concentration of $2.2\,g\,DHGLA\,l^{-1}$ (Ogawa et al. 2002), while desaturase-defective mutants of *M. alpina* produce relatively high amounts of DHGLA, without the use of inhibitors.

2.2.4 Other Derived Fatty Acids

There is a series of mutants of *M. alpina*, which are able to synthesise rare fatty acids. One of them produces 5,8,11-eicosatrienoic acid called Mead acid (Kawashima et al. 1997), which is known to be a precursor of LT_3 leukotrienes and a reductant of inflammatory diseases. This fatty acid is formed via sequential conversion of oleic acid, involving Δ^6 desaturation, elongation, and finally Δ^5 desaturation (Certik and Shimizu 1999). When grown with linseed oil, a Δ^5/Δ^{12} desaturase-defective mutant of *M. alpina 1S4* produced up to $23\,g\,L^{-1}$ 8,11,14,17 cis-eicosatetraenoic acid (C20:4 ω-3) by the 12th day (Kawashima et al. 1998).

Saprolegnia sp. *28YTF*-1 was found to accumulate odd chain PUFAs of carbon chain length 17 to 19, when grown with fatty acids having 13, 15, or 17 carbons (Shirasaka and Yokochi 1995). This suggests that, by feeding fungi with the appropriate precursor when they are grown with adequate cultural conditions, biosynthesis of very specific fatty acids is feasible.

2.2.5 Docosahexaenoic Acid

This ω-3 fatty acid (C22:6 cis 4,7,10,13,16,19) is added with AA to infant formula (Gunstone 1999). The main source of DHA is fish oil, which may have an objectionable taste and odor and from which cholesterol must be removed (Certik and Shimizu 1999). Alternative sources are two algae: *Crypthecodinium cohnii* produces DHA as its sole polyunsaturated fatty acid (Ratledge et al. 2001), while *Isochrysis galbana* is under investigation for DHA production (Poisson and Ergan 2001). The sole fungus reported to contain significant amounts of DHA is the marine Chytrid *Traustochytrium roseum*, several strains of which can accumulate up to 50% of this fatty acid in oil (Singh and Ward 1996; Singh et al. 1996) but, unfortunately, its oil content is low (Certik and Shimizu 1999). The closely related *Schizochytrium* SR21 may produce up to $15.5\,g\,L^{-1}$ DHA per day (Nakahara et al. 1996). The search for better sources produced a series of strains of *Thraustochytrium* (Huang et al. 2001), among which 3 groups could be recognized, producing respectively as percent of total FA:

DPA + DHA (strain KH 105): DHA = 46.9%
DHA/DPA/EPA: strains KH154 (DHA = 58.5%) and KH155 (DHA = 59.7%)
DHA/DPA/EPA/AA: strain KK17-3 (DHA = 52.1%)

An additional series includes 2 strains, which produce C22:4n-6 in addition to DHA/DPA/EPA/AA (Huang et al. 2001). These new strains present suitable characteristics for industrial utilization. *Geotrichum* sp. FO347-2, a filamentous fungus, may use refined sardine oil (12.3% DHA) as sole C source. If DHA from this oil is concentrated in cellular lipid of this *Geotrichum* sp., the content is 25.9% after 24 h. and may reach $55\,mg\,g^{-1}$ of d.w. when using tuna head oil (26.8 % DHA). In this case, the recovery rate of DHA from the tuna oil is 19.7% (Kim et al. 1999). All of these new strains could lead to industrial production of DHA.

3 MOLECULAR BIOLOGY

Saccharomyces cerevisiae has been the most frequent tool for investigation of lipid metabolism at the molecular level and the genetic manipulation of fungal lipid composition. The expression of yeast fatty acid synthetase genes (Scheuller et al. 1990) and the regulation of the Δ^9 fatty acid desaturase gene were widely studied (Huang et al. 1991). The trifunctional fatty acid synthetase gene FAS2 encoding subunit alpha of the yeast fatty acid synthetase complex was mapped by three independent methods (Siebenlist et al. 1990).

Budziszewski et al. (1996) reviewed molecular biology methods, which can be used for modifying plant lipids with the initial aim of increasing the erucic acid content of rapeseed oil because this fatty acid is used for nylon synthesis. Since certain fungi are able to produce highly valuable PUFAs, research focused widely on desaturases genes which were cloned, leading to the production of series of mutants (Certik et al. 1998).

3.1 Use of Selected Mutants

Arachidonic acid (C20:4n-6) is synthesized by the Δ^5-desaturation of dihomo-γ-linolenic acid (C20:3). The Δ^5-fatty acid desaturase gene was first isolated from *Mortierella alpina*, then its function was confirmed by

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expression in *S. cerevisiae* (Michaelson et al. 1998) and also in canola (Knutzon et al. 1998). Dihomo-γ-linolenic acid was industrially produced by the Δ^5 desaturase-defective mutant 1S-4 of *Mortierella alpina* (Kawashima et al. 2000). The first nonplant Δ^{12}-desaturase was cloned from this strain (Sakuradani et al. 1999a). A *M. alpina* 1S-4 mutant, defective in Δ^5, Δ^{12} desaturases, accumulated 8,11-cis eicosadienoic acid (C20:2n-9) instead of arachidonic or dihomo-γ-linolenic acids (Kamada et al. 1999). For the synthesis of arachidonic acid, desaturations are combined with an elongation of 18:3n-6 to 20:3n-6, which can be the limiting step in this pathway (Wynn 2000). Jareonkitmongol et al. (1994) isolated an ω-3-desaturation defective mutant of this 1S-4 strain of *M. alpina*.

3.2 Heterologous Expression of Desaturases

Crucial desaturases, which have been isolated from certain fungi can be transferred to other fungi. This is of interest if the donor strain is not a good lipid producer. Transferring the right specific gene into an "oleaginous" fungus should lead to qualitative modification of the fatty acid content of this latter species. cDNAs encoding Δ^6 and Δ^{12}-desaturases from *M. alpina* were transferred into *S. cerevisiae*, a species which is easier to grow. Expression of the Δ^{12}-desaturase cDNA led to the production of linoleic acid. The Δ^6-desaturase activity may allow to the production of γ-linolenic acid provided the culture medium had been supplemented with linoleic acid. Coexpression of both the Δ^6 and Δ^{12}-desaturases resulted in the endogenous production of γ-linolenic acid by *S. cerevisiae* (Huang et al. 1999). In the same way, a Δ^9 desaturase cDNA from an arachidonic acid-producing *M. alpina1S-4* could be expressed in *Aspergillus oryzae*, resulting in drastic changes in the fatty acid composition of the transformant cells (Sakuradani et al. 1999b). Kelder et al. (2001) obtained the expression of fungal desaturases genes in cultures of mammalian cells, showing the feasibility of generating cell lines, which no longer require dietary "essential" fatty acids by altering the endogenous fatty acid metabolism to enhance the production of long-chain PUFAs.

4 SURFACTANTS

4.1 Biosurfactant Glycolipids

Fungal glycolipids of various degrees of complexity are increasingly of interest as emulsifiers in food and drug industries as well as in bioremediation of hydrocarbon or fatty acid pollution in soils (Kitamoto 1992; Sarubbo et al. 1999). The surfactant properties of these complex lipids, reflects their molecular structure, in which hydrophobic moieties, including a diversity of fatty acids, are attached to hydrophilic moieties, such as carbohydrates, amino acids, lipoproteins, lipopeptides, phospholipids, esters, etc. A "presumptive biosynthetic pathway" for one class of such compounds, mannosylerythritol lipid (MEL) in *Candida antarctica*, published by Kitamoto et al. (1998), illustrates the metabolic complexity involved in biosurfactant production. A glycolipid biosurfactant from another species *Candida* sp. SY16 was shown by Kim et al. (1999) to contain mannosylerythritol, as hydrophilic moiety, and fatty acids (C 16:0, C 22:0, C 24:0, and C 24:1) as hydrophobic moiety, with an acetal group linked to C 6 of D-mannose in the hydrophilic moiety.

4.2 Production of Fungal Surfactants

Biosurfactant production by *C. antarctica* has been studied for more than a decade by Kitamoto et al. (1992b). Growing on glucose or other carbohydrates as sole carbon source, over 10% of its dry weight was found in intracellular oil globules containing mainly TAG and MEL (Kitamoto et al. 1992a). Resting cells produced 20% more extracellular mannosyl-erythritol lipid from than growing cultures, yielding 47 $g\,L^{-1}$ after 6 days of shake culture in media containing only a water-insoluble carbon source (Kitamoto et al. 1992b) and rose to 80 $g\,L^{-1}$, following further addition of peanut oil and incubation for another 6 days, with the advantage of easier extraction and the possibility of convenient recovery of biosurfactants in an immobilized reactor system. The antimicrobial activity of mannosyl-erythritol lipids from *C. antarctica* growing on soybean oil have also been examined particularly against Gram positive bacteria, quantified (Kitamoto et al. 1993).

Despite their low toxicity, biodegradability and, in some cases, specific medical and pharmacological value, biosurfactants can only compete economically with conventional chemical alternatives if obtained from fermentations based on inexpensive, renewable carbon sources, or by-products from food processing. However, biotechnological exploitation can also confer advantages in removing the significant environmental hazards associated with the accumulation of such materials, as shown in the biosynthesis of extra- and intracellular lipid by *Candida bombicola* growing on cheese whey (Zhou and Kosaric 1993), particularly in later exponential phase, during nitrogen depletion, and continuing into the stationary phase. Using animal fat from meat processing waste, Deshpande and Daniels (1995) obtained efficient production of biosurfactant lipid (120 $g\,L^{-1}$ in 68 h) by *C. bombicola* as well as a final yield of good quality single cell protein, on medium containing animal fat and glucose as carbon source, corn steep liquor as vitamin source, plus ammonium sulfate, potassium phosphate, and urea. Growing on glucose (15%) plus 2-dodecanol (1.5%), the same species produced novel biosurfactant alkyl glycosides (22 $g\,L^{-1}$), with glucose and sophorose as carbohydrate moieties and 2-dodecanol as the major lipid moiety (Brakemeir et al. 1998). With *C. bombicola* ATCC22214, Guilmanov et al. (2002) recorded optimal production of surfactant sophorose lipids (350 $g\,L^{-1}$) at oxygen transfer rates between 50 and 80 mM $O_2\,L^{-1}\,h^{-1}$.

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4.3 Manipulation of Fermentation for Surfactants

As mentioned above for *C. antarctica* and also demonstrated with *Candida apicola* by Hommel et al. (1994), glycolipid synthesis is depressed by excess nitrogen in fermentation media. Similarly, production of surfactant sophorose lipids by *C. bombicola* when growth on glucose and rapeseed oil, was nitrogen-limited and on further addition of lipid substrate rose to $250-300\,g\,L^{-1}$ (Davila et al. 1997). In this respect, glycolipid production resembles secondary metabolism, as pointed out by Sarubbo et al. (1999) in the case of production of a surfactant polysaccharide-protein-lipid complex by *Candida lipolytica* from babassu oil, which also occurred in cultures growing on glucose as sole carbon source, reaching a maximum in stationary phase (Sarubbo et al. 2001).

It is striking that, although many other fungal genera and species are known to produce surfactants, the industrial production processes developed so far have concentrated on a small number of species from a single genus. This is understandable when *Candida* species have proved so amenable to conventional industrial fermentation processes. However many other taxa are worthy of investigation for novel surfactants, even if some of these may require techniques of immobilized culture or solid state fermentation to show their full metabolic potential.

5 FUNGAL LIPASES

Geotrichum candidum, *Candida cylindracea*, *Rhizomucor miehei*, and *Rhizopus delemar* produce lipases which are commercially available. Some of these enzymes have been widely studied (Sidebottom et al. 1991) and exhibit interesting properties, such as selectivity towards either specific fatty acids (Haas et al. 2000) or the esterified position on the glycerol molecule (Baillargeon and McCarthy 1991; Sugihara et al. 1991). However, many other fungi also produce lipases (see Sancholle and Lösel 1995 or Weete and Gandhi 1992).

Since it is well known that the availability of a particular fatty acid depends on its position on the glycerol molecule, the special properties of these fungal lipases can be exploited to improve the digestibility or nutritional properties of oils (Valenzuela and Nieto 1994) by allowing esterification of the appropriate position of the glycerol molecule with the most suitable fatty acid (Shimada et al. 1999). Thus, structured glycerides can be built up by using fungal lipases for transesterification, acidolysis, or hydrolysis. The specificity of some fungal lipases for given fatty acids may lead to their use for the preparation of pure fatty acids, particularly PUFAs, by selective hydrolysis of the oils of which they are constituents (Shimada et al. 1997). For this they can be used as either free or adsorbed enzymes and also in aqueous or organic solvents (Kosugi et al. 1997; 2000; Lee and Parkin 2001).

5.1 Oil Modifications

Borage, melon seed, and fish oils are known to be very rich in PUFAs. Their nutritional value may be improved by the incorporation of some medium-chain fatty acids like capric acid (C10:0). The lipase of *Rhizomucor miehei* was successfully used by Jennings and Akoh (1999, 2001) for the incorporation of capric acid into fish oil. The composition of menhaden fish oil was greatly modified by incorporation of 30% of capric acid together with a decrease of C20:5 (17%) and C 22:6 (14%). Borage oil was restructured by using the lipases of *C. antarctica* (SP 435) and *R. miehei* (IM 60); the transesterification reactions leading to an incorporation of capric acid (C10:0) and EPA (C20:5 n3). Better results were obtained with IM60 (sn-1,3 specific) from *R. miehei* than with the nonspecific SP 435 lipase from *C. antartica* (Akoh and Moussata 1998). These lipases were also used successfully for the incorporation of *n*-3 PUFAs into melon seed oil (Huang et al. 1994), in which the rate of incorporation was better with EPA ethyl ester than with free EPA; moreover IM60 requires additional water whereas SP435 is totally inhibited by any trace of water. Fats enriched with ω-3 PUFAs were prepared by transesterification of palm oil with a concentrate of triglycerides enriched with ω-3 PUFAs and soybean oil, using a commercial immobilized lipase of *C. antarctica* as catalyst (Osorio et al. 2001).

The treated oils were randomly enriched with PUFAs, which gave them better nutritional properties. Under adequate reaction conditions, specific lipases may allow the preparation of structured lipids with selected fatty acids on chosen positions of the glycerol molecule.

5.2 Preparation of Structured Glycerides

Interesterification of tricaprin and trilinolein with immobilized lipases IM60 (*R. miehei*) and SP 435 (*C. antarctica*) as biocatalysts allowed the preparation of structured triglycerides containing either one (SL1) or two (SL2) linoleic acids per triacyglycerol molecule. When SP 435 was used, the fatty acid at the sn-2 position was 43.6 mol% capric acid and 56.4 mol% linoleic acid, while that from SL2 contained 56.6 mol% capric acid and 43.4 mol% linoleic acid (Lee and Akoh 1997).

Hydrolysis of coconut oil by a nonspecific lipase from *Candida cylindracea* provided good amounts of lauric acid, which was used by Nieto et al. (1999b) for the production of structured sn-1, sn-3 dilauryl, sn-2 eicosapentaenoyl glycerol and sn-1, sn-3 dilauryl, sn-2 docosahexaenoylglycerol by transesterification of sn-2 eicosapentaenoyl glycerol and sn-2 docosahexaenoyl glycerol. The required monoglycerides had been prepared by hydrolyzing fish oil with the sn-1, sn-3 specific immobilized lipase Lipozyme IM20 from *Mucor miehei* (Nieto et al. 1999a). A structured lipid containing gamma-linolenic acid (GLA) was synthesized by continuous acidolysis of borage oil with caprylic acid, using 1,3-specific

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R. delemar lipase as a catalyst. The lipase was immobilized on a ceramic carrier (Shimada et al. 1999). Lipozyme IM20, a 1,3-specific immobilized lipase from *Rhizomucor miehei* was used in a packed bed bioreactor for the preparation of structured triacylglycerols from canola oil with caprylic acid as the acyl donor (Xu et al. 2000).

A 1,3-positionally specific thermostable lipase (R275A lipase) from *Fusarium heterosporum* was recently developed. When immobilized on Dowex WBA, this lipase gave excellent performances for the preparation of 1,3-dioleoyl-2-palmitoyl-glycerol, 1(3),2-dioleoyl-3(1)-palmitoyl-glycerol, 1(3),2-palmitoyl-3(1)-oleoyl-glycerol and 1,3-dipalmitoyl-2-oleoyl-glycerol, from a mixture of tripalmitoyl-glycerol/oleic acid (1:2 w/w). The half-life of this new lipase (370 days) was much longer than the half-life of lipases from *R. delemar* or *R. miehei* used in similar conditions (Nagao et al. 2001).

Even the production of good quality solid fats may be achievable by the use of fungal lipases. Seriburi and Akoh (1998) prepared a solid fat with the properties of soft-type margarine by interesterifying lard and high-oleic sunflower oil with the SP435 lipase from *C. antarctica*.

5.3 Other Uses of Fungal Lipases

Phospholipase A-mediated degumming is a well-established process step in physical refining of vegetable oils. Lecitase® Novo, an A1 type phospholipase from *F. oxysporum*, which was used for this purpose by Clausen (2001), has proved to be superior to porcine pancreatic Lecitase® 10L and other phospholipases.

6 CONCLUSION

The above survey reveals striking variation in rates of progress in the area of fungal lipid biotechnology discussed, according to the current levels of interest in the various products. The outstanding ùedical and dietary significance of PUFAs et eicosapolyenoic acids, which can be obtained uniquely from the diversity of fungal metabolism is matched by the exploitation of a relatively wide range of fungi. In contrast, fewer genera and species have been employed in the industrial production of fungal lipases, although the awareness of their potential in the remodeling of valuable lipid is expanding very rapidly. As noted in the short section of the production of biosurfactants, although a wider range of fungal sources has long been recognized, industrial processes basically employ a limited number of species from the single genus *Candida*.

Despite the obvious economic temptations for biotechnological industries to rely on a small number of organisms, the behavior of which in industrial fermentation conditions is relatively well understood, an awareness has to be maintained of the enormous untapped metabolic potential of the diversity of fungal taxa and of the need for greater development of immobilized or solid-state fermentation systems, more favorable to their growth and expression of this potential. The recent progress in the molecular biology of fungal lipids, discussed above, points also to new possibilities in further extending the range of fungal biosynthetic mechanisms available into more easily cultured system.

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27

Introduction to the Theory of Metabolic Modeling and Optimization of Biochemical Systems: Application to Citric Acid Production in *Aspergillus niger*

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1 INTRODUCTION

Biochemistry attempts to understand and control physiological events through detailed studies of the chemical constituents of living matter. The most frequent, classical modus operandi of biochemistry is *reductionistic*. It calls for a reduction of a macroscopic system to the elemental structures and properties of its microscopic constituents, with the hope that detailed information about all parts would lead to comprehension of the functioning as a whole. It is becoming increasingly apparent that this reductionistic approach, while being extremely successful in its own right, does not suffice to decipher the internal logic of complex biochemical systems. Knowledge of the properties of enzymes, proteins, and other metabolites alone is not sufficient to explain organismic functioning, because interactions among the biochemical constituents lead to systemic behaviors that are not evident, or not even existent, in any of the constituents themselves. Even a very detailed analysis of all glycolytic enzymes and metabolites in isolation is not likely to predict or explain the regular oscillations that have often been observed in glycolysis (Goldbeter 1996).

The complexities and intricacies of metabolic pathways, and the reconstruction that must accompany reductionism if we target a deeper understanding at the systemic level, require novel types of tools that are different from the classical experiments of biochemistry. We need tools of integration, tools that allow a full, quantitative account of the numerous constituents and their interactions that we encounter in metabolic and gene regulatory networks. Given the size and complexity of these networks *in vivo*, these tools must be of a mathematical nature. Mathematical models provide us with precise, objective, and unambiguous representations of knowledge. Mathematical modeling allows us to organize and merge diverse pieces of available information. It facilitates very large bookkeeping tasks that immediately arise if systems have a realistic size. Only mathematics has the tools for quantitatively specifying the structure and dynamics of interconnected processes, which the unaided human mind is simply unable to do. Mathematical modeling allows us to test the significance and validity of data and assumptions and offers objective guidance in decision-making and experimental design.

Mathematical modeling is a comparatively young field, and there are no generally accepted guidelines for what constitutes a good model. It is clear that a good model should be valid and have explanatory and predictive power. In addition, it is being realized more and more that a model facilitates insight only if it has a mathematical structure that is simple enough for streamlined algebraic and computational analysis. The significance of issues of validity on one hand, and of mathematical and computational tractability on the other, becomes evident when we consider what makes natural metabolic networks work. First, they contain many substrates, intermediate metabolites, products, enzymes, cofactors, and modulators that need to be tracked over time. Secondly, enzyme-catalyzed steps have nonlinear characteristics, which prevent us from using simple scaling arguments: Doubling

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the substrate concentration usually does not lead to twice the reaction rate or flux. Similarly, activators and inhibitors affect reactions in a nonlinear fashion. Thirdly, the synergisms and antagonisms among processes and pathways may lead to oscillations, limit cycles, or even chaotic dynamics [e.g., Goldbeter (1996); Olsen and Degn (1977)]. All these phenomena are found in living systems, which imply that a valid mathematical description of a metabolic system must be able to account for them. The aim of this chapter is to introduce some basic concepts of a metabolic modeling framework that is able to deal with these requirements and allows insight, prediction, control, and optimization. We will illustrate this framework with a specific application, namely the analysis and optimization of citric acid production in the mould *Aspergillus niger*.

2 BIOCHEMICAL SYSTEMS THEORY: A POWERFUL THEORETICAL FRAMEWORK FOR MODELLING BIOCHEMICAL SYSTEMS

A modeling approach that fully acknowledges the complexity and nonlinear nature of metabolic networks is based on a mathematical framework called *Biochemical Systems Theory* (BST; Savageau 1969a, b; 1970). The hallmark of this theory is the approximation of rate laws and other processes with products of power-law functions. Though this type of representation, in the beginning, may be unfamiliar and odd, it is mathematically very convenient. It is also solidly grounded in the well-established theorem of Brook Taylor (1685–1731), which is the foundation of modern approximation theory.

2.1 Introduction to the Power-Law Representation

Let's consider a simple Michaelis–Menten mechanism, in which substrate X is converted into product P via an enzyme-catalyzed reaction:

$$X \xrightarrow[v]{V_{\max}, K_M} P$$

The corresponding rate law takes the well-known form:

$$v = v(X) = \frac{V_{\max} X}{K_M + X},$$

where K_M and $V_{\max}$ are the Michaelis constant and the maximal velocity of the reaction, respectively. In the power-law formalism, the same reaction is represented by the power-law function

$$\tilde{v} = \alpha \cdot X^g.$$

The exponent, g, is called *kinetic order*, and the multiplier, α, is called *rate constant*. These parameters can be measured in the lab, if the rate $\tilde{v}$ is measured for several substrate concentrations X; details will be given later.

While the power-function rate law is a representation in its own right, and while it is entirely independent of other traditional formulations, it can also be considered an approximation, for instance, of the traditional Michaelis–Menten rate law. As a "local" approximation, $\tilde{v}$ exactly coincides with the approximated rate v for one substrate concentration, say X_0, and gives very similar values of the rate for other substrate concentrations close by. The relationship between $\tilde{v}$ and v is given through the values of the kinetic order and the rate constant. Specifically, the kinetic order, g, is the derivative of the logarithm of the function v with respect to the logarithm of the variable X,

$$g = \frac{d \ln v}{d \ln X} = \frac{dv}{dX} \cdot \frac{X}{v}$$

and this expression is to be evaluated at X_0. The rate constant is obtained as

$$\alpha = \frac{v(X_0)}{(X_0)^g}$$

which holds because $\tilde{v}$and v by design are equal for the substrate concentration of choice, X_0. This concentration of choice is often the concentration found in the cell or organism under undisturbed, natural conditions. In mathematical terminology, it is called the "operating point."

Thus, while the power-law formulation can be developed and measured without consideration of the traditional Miachelis–Menten mechanism, it can be considered as its approximation, and this approximation is characterized by the operating point, a real-valued kinetic order, and a nonnegative rate constant. The two parameters of the power-law rate, if considered as an approximation, are numerically dependent on the operating point. If the operating point is moved from zero toward larger substrate concentrations, the kinetic order g that models a Michaelis–Menten rate decreases concomitantly from 1 to 0. Notably, for $X_0 = K_M$ the kinetic order is 0.5, which can be deduced directly from the above formula of g [see also Voit (2000)].

A crucially significant benefit of the power-law approach is that it translates directly into higher dimensions. If many metabolites or other factors contribute to a rate V, this rate is a function of all of them. In mathematical terminology, we thus consider the rate $V(X_1, X_2, X_3, X_4, \ldots, X_n)$. This rate is directly expressed in the power-law formalism as a product of power-law functions: $V = \alpha \cdot X_1^{g_1} X_2^{g_2} X_3^{g_3} X_4^{g_4}, \ldots, X_n^{g_n}$. Only those variables that directly affect V enter this power-law representation. If a variable X_k does not affect V directly, the derivative of V with respect to X_k is zero. Consequently, g_k is zero and the expression $X_k^{g_k}$ equals 1, regardless of the value of X_k. The product structure of the representation makes it clear that all such factors $X_k^{g_k} = 1$ are irrelevant and may be omitted.

Each kinetic order g_i can be directly calculated through *partial* differentiation of V with respect to X_i in

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a fashion that is directly analogous to the computation of g above. X_1, X_2, and X_3 correspond to the dependent variables that are represented by three differential equations. Similarly, once all kinetic orders have been estimated, the rate constant for a multivariate function is computed from the equivalence of the power-law representation and the approximated rate function at the operating point of choice. Thus, one computes straightforwardly $\alpha = V \cdot X_1^{-g_1} X_2^{-g_2}, \ldots, X_n^{-g_n}$, which again is evaluated at the operating point.

While the numerical values of kinetic orders and rate constants change with the choice of the operating point, the structure of the approximation is always the same, namely that of a power-law function of the type αX^g or $\alpha \cdot X_1^{g_1} X_2^{g_2} X_3^{g_3} X_4^{g_4}, \ldots, X_n^{g_n}$. This implies that a rate law may be set up symbolically without knowledge of the exact structure of the underlying process.

2.2 S-Systems

The power-law representation is the basis of a general system description, which, in turn, is the foundation of BST [for detailed accounts, see Savageau (1976); Torres and Voit (2002); Voit (1991; 2000)]. This system description consists of a differential equation for each variable that changes over time, and the right-hand side of each differential equation is defined as the difference of two products of power-law functions, one representing all contributions to the production of the variable and one representing all contributions to the degradation of the variable. This formulation is called an *S-system*. One may wonder how general this type of formulations is. Surprisingly, Savageau and Voit (1987) proved that virtually any differentiable nonlinearity can be represented exactly by an S-system.

As a generic example for illustration purposes, consider the simple branched pathway in Figure 1. In this pathway, X_4 is provided from the outside and yields X_1, which, as the substrate of interest, is used for the production of X_2 and X_3. Product X_2 inhibits the synthesis of X_1. To obtain the S-system description, we formulate the dynamics with a set of differential equations, each one representing the change in one dependent variable X_i. Because X_4 in our example is considered a constant external supply, it is modeled as an *independent* variable, which does not require a differential equation of its own, because it does not change during any given experiment; X_4 may differ from one experiment to other, thereby allowing us to model different input scenarios.

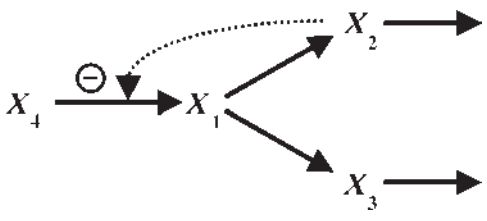

Figure 1 Simple branched pathway with inhibition. X_4 is considered an independent (external) variable that provides constant influx to the pathway.

The symbolic S-system formulation of the pathway is constructed directly by setting up power-law terms and including those terms that directly affect a given term:

$$\frac{dX_1}{dt} = V_1^+ - V_1^- = \alpha_1 X_2^{g_{12}} X_4^{g_{14}} - \beta_1 X_1^{h_{11}}$$

$$\frac{dX_2}{dt} = V_2^+ - V_2^- = \alpha_2 X_1^{g_{21}} - \beta_2 X_2^{h_{22}}$$

$$\frac{dX_3}{dt} = V_3^+ - V_3^- = \alpha_3 X_1^{g_{31}} - \beta_3 X_3^{h_{33}}$$

$$X_4 = \text{constant}$$

Each function V_i^+ represents all effects that influence the production of X_i, and each function V_i^- represents all effects that influence the degradation of X_i, for $i = 1, 2, 3$. A prominent feature to note about these functions is their homogeneous structure. A variable, whether dependent or independent, is formally treated in the same fashion, namely, as a factor in the product V_i^+ or V_i^-, raised to a power. This formulation is very different from conventional rate laws, which usually take the form of rational functions, and in which substrates and modulators appear in different forms.

One may wonder about the degradation of X_1, which occurs through two pathways. Indeed, this process is described by three terms, namely $\beta_1 X_1^{h_{11}}$, $\alpha_2 X_1^{g_{21}}$, and $\alpha_3 X_1^{g_{31}}$. At the operating point of choice, these terms relate as $\beta_1 X_1^{h_{11}} = \alpha_2 X_1^{g_{21}} + \alpha_3 X_1^{g_{31}}$, which reflects that the total degradation equals the sum of the two production steps toward X_2 and X_3. For a detailed discussion, see Voit (2000).

Because of the rigid structure of these types of models, which is combined with great flexibility in capturing nonlinear phenomena, S-systems and related formulations have been called "canonical models" (Savageau and Voit 1987; Voit 1991) The most significant advantage of a canonical model is the fact that its homogeneous structure allows the standardization of a typical analysis, which includes model design, parameter estimation, steady-state, stability and robustness analysis, and dynamical exploration.

3 APPLICATION OF BST TO THE ANALYSIS AND OPTIMIZATION OF *ASPERGILLUS NIGER* METABOLISM UNDER CONDITIONS OF CITRIC ACID PRODUCTION

3.1 Model Design and Mathematical Representation

Based on a comprehensive body of kinetic and biochemical information about *A. niger*, and using the above modeling concepts, it is possible to design an integrated S-system model

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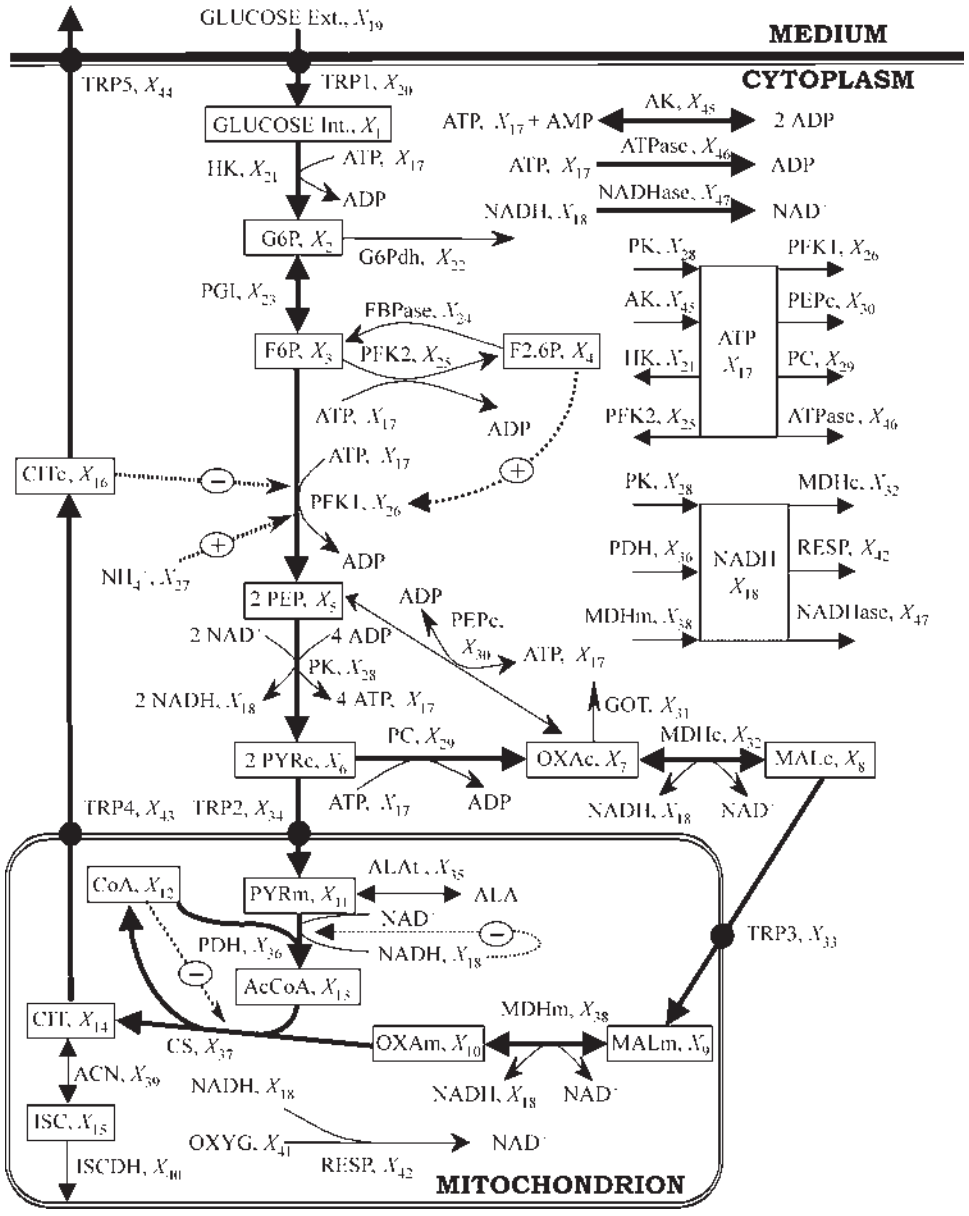

Figure 2 Metabolic map of citric acid metabolism in *A. niger*. Dependent variables are numbered from 1–18: Glucose Int. (X_1, cytosolic glucose); G6P (X_2, glucose 6-phosphate); F6P (X_3, fructose 6-phosphate); F2,6P (X_4, fructose 2,6-bisphosphate); PEP (X_5, phosphoenolpyruvate); PYRc (X_6, cytosolic pyruvate); OXAc (X_7, cytosolic oxalacetate); MALc (X_8, cytosolic malate); MALm (X_9, mitochondrial malate); OXAm (X_{10}, mitochondrial oxalacetate); PYRm (X_{11}, mitochondrial pyruvate); CoA (X_{12}); AcCoA, (X_{13}, acetyl-CoA); CITm (X_{14}, mitochondrial citrate); ISC (X_{15}, isocitrate); CITc (X_{16}, cytosolic citrate); ATP (X_{17}); NADH (X_{18}). Independent variables are numbered from 19–49: Glucose Ext. (X_{19}, glucose in medium); TRP1 (X_{20}, glucose carrier); HK (X_{21}, hexokinase); G6Pdh (X_{22}, glucose 6-phosphate dehydrogenase); PGI (X_{23}, phosphoglucose isomerase); FBPase (X_{24}, fructose 2,6-bisphosphatase); PFK2 (X_{25}, 6-phosphofructo-2-kinase); PFK1 (X_{26}, phosphofructokinase); NH_4^+ (X_{27}, ammonium); PK (X_{28}, pyruvate kinase); PC (X_{29}, pyruvate carboxylase); PEPc (X_{30}, cytosolic phosphoenolpyruvate carboxykinase); GOT (X_{31}, aspartate aminotransferase); MDHc (X_{32}, cytosolic malate dehydrogenase); TRP3 (X_{33}, mitochondrial malate transport system); TRP2 (X_{34}, mitochondrial pyruvate transport system); ALAt (X_{35}, alanine transaminase); PDH (X_{36}, pyruvate dehydrogenase); CS (X_{37}, citrate synthase); MDHm (X_{38}, mitochondrial malate dehydrogenase); ACN (X_{39}, aconitase); ISCDH (X_{40}, isocitrate dehydrogenase); OXYG (X_{41}, oxygen); RESP (X_{42}, alternative respiratory system); TRP4 (X_{43}, mitochondrial citrate carrier); TRP5 (X_{44}, cytosolic citrate carrier); AK (X_{45}, adenylate kinase); ATPase (X_{46}); NADHase (X_{47}, dehydrogenases).

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of citric acid production that accounts for all cellular processes that are considered important in the present context. Of particular interest for questions of citric acid yield optimization is the idiophase steady state (at 150 h of cultivation), where most of the metabolic activity in *A. niger* consists of citric acid accumulation. The biochemical information during this phase is first presented as a description of the main regulatory features of the system and subsequently summarized in the form of a biochemical map.

Five metabolic processes play a leading role in the citric acid biosynthesis of *A. niger*: (a) breakdown of hexoses to pyruvate and acetyl-CoA during glycolysis, (b) citric acid formation within the tricarboxylic acid cycle, (c) anaplerotic formation of oxalacetate from pyruvate and CO_2, (d) transport processes throughout the cytoplasmatic and mitochondrial membranes, and (e) oxygen consumption.

An extensive review of the literature provided quantitative experimental evidence about these processes, as well as kinetic data on metabolite pools, fluxes, and enzyme levels. This information was arranged in the metabolic map shown in Figure 2. Mechanistic and kinetics details are found in Alvarez-Vasquez (2000), Alvarez-Vasquez et al. (2000), and Kubicek et al. (1979).

The symbolic S-system representation is directly derived from the metabolic map in Figure 2, by establishing equations for all dependent variables that consist of one production and one degradation term each. Each of these terms has the form of a product of power-law functions, as outlined above. For instance, the equation for cytosolic glucose (X_1) reads in symbolic form

$$\dot{X}_1 = \alpha_1 X_{19}^{g_{1,19}} X_{20}^{g_{1,20}} - \beta_1 X_1^{h_{1,1}} X_{17}^{h_{1,17}} X_{21}^{h_{1,21}} \quad (1)$$

The production is governed by the amount of external glucose

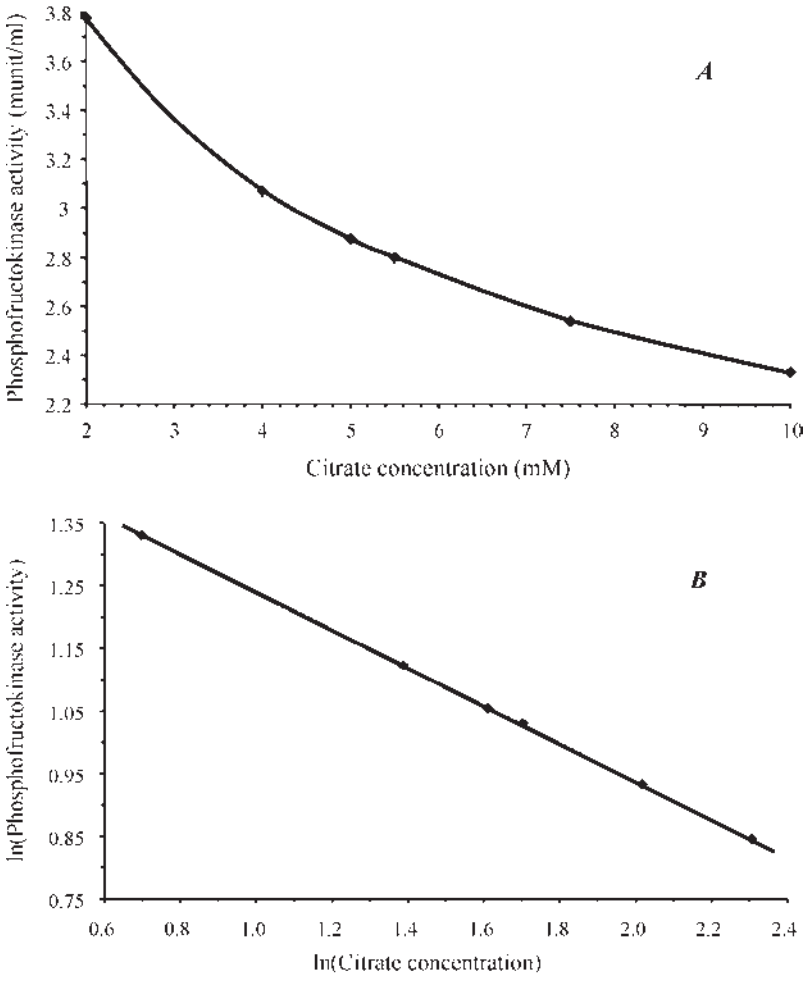

Figure 3 *Aspergillus niger* phosphofructokinase activity decreases with increasing citrate concentration. Panel A shows the original data in Cartesian coordinates. Representation in logarithmic coordinates (Panel B) provides the corresponding kinetic order directly as the slope of the graph. Data from Habinson et al. (1983).

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(X_{19}) and the activity of the glucose carrier TRP1, which is coded as X_{20}. The degradation depends of the cytosolic glucose concentration itself (X_1), on ATP (X_{17}), and on the activity of hexokinase (X_{21}). Kinetic orders and rate constants are included and indexed as specified by the rules of BST. In this fashion, equations for all 18 dependent variables are deduced directly from the map in Figure 2.

3.2 Parameter Estimation and Numerical Representation

Kinetic orders for the S-system model can be estimated from flux and concentration measurements, published rate functions, or a variety of specific experiments [for details see Voit (2000)]. As an example of the latter case, measurements of the reaction rate v are plotted against a series of concentrations of a substrate or modulator variable X_k, while all other variables are kept at the same level. Such a plot is typically curved, but often becomes almost linear, when concentration and rate measurements are plotted on logarithmic axes. In this logarithmic plot, the kinetic order is directly given as the slope of the relationship between rate and metabolite concentration at the operating point. An example is shown in Figure 3, where phosphofructokinase activity is plotted against citrate concentration.

In this fashion, but more often by deriving parameters from published rate functions, all parameters of the S-system model were measured or computed (Alvarez-Vasquez 2000; Alvarez-Vasquez et al. 2000). The result is a fully parameterized, numerical S-system representation that has the following form:

$$\dot{X}_1 = 0.1857 \cdot X_{19}^{0.0124} X_{20} - 54.4794 X_1^{0.9915} X_{17}^{0.5798} X_{21}$$

$$\dot{X}_2 = 50.5542 \cdot X_1^{0.976} X_2^{-0.026} X_3^{0.04167} X_{17}^{0.5707} X_{21}^{0.9843} X_{23}^{0.0156} - 0.0018 \cdot X_2^{2.202} X_3^{-1.3389} X_{22}^{0.1968} X_{23}^{0.8031}$$

$$\dot{X}_3 = 0.0019 \cdot X_2^{2.2316} X_3^{-1.3956} X_4^{0.1604} X_{23}^{0.8371} X_{24}^{0.1628} - 0.6254 \cdot X_2^{-0.0271} X_3^{0.6208} X_4^{0.001} X_{16}^{-0.2462} X_{17}^{0.1212} \times X_{23}^{0.0162} X_{25}^{0.1628} X_{26}^{0.8208} X_{27}^{0.5335}$$

$$\dot{X}_4 = 0.2462 \cdot X_3^{0.96} X_{17}^{0.5833} X_{25} - 0.9767 \cdot X_4^{0.9852} X_{24}$$

$$\dot{X}_5 = 1.023 \cdot X_3^{0.5004} X_4^{0.0012} X_5^{-0.0016} X_7^{0.026} X_{16}^{-0.2926} X_{17}^{0.0683} \times X_{26}^{0.9756} X_{27}^{0.6341} X_{30}^{0.0243} - 1.6341 \cdot X_5^{0.9107} X_7^{-0.0007} \times X_{17}^{-3.2606} X_{18}^{-0.0047} X_{28}^{0.9893} X_{30}^{0.0106} X_{48}^{3.7481} X_{49}^{0.1014}$$

$$\dot{X}_6 = 1.6565 \cdot X_5^{0.909} X_{17}^{-3.2183} X_{18}^{-0.0047} X_{28} X_{48}^{3.7011} X_{49}^{0.1025} - 2.644 \cdot X_6^{0.835} X_{17}^{0.1636} X_{29}^{0.5193} X_{34}^{0.4807}$$

$$\dot{X}_7 = 1.3835 \cdot X_5^{0.0212} X_6^{0.6733} X_7^{-0.015} X_8^{0.0318} X_{17}^{0.1598} X_{18}^{-0.0152} \times X_{29}^{0.9619} X_{30}^{0.0199} X_{32}^{0.0181} X_{48}^{0.1618} X_{49}^{0.0196} - 1.7805 \times 10^5 \cdot X_5^{-0.0031} X_7^{1.6839} X_8^{-0.6832} X_{17}^{0.0694} \times X_{18}^{1.618} X_{30}^{0.0456} X_{31}^{0.0507} X_{32}^{0.9035}$$

$$\dot{X}_8 = 3.5235 \times 10^5 X_7^{1.7538} X_8^{-0.7561} X_{18}^{1.7906} X_{32} - 0.0033 \cdot X_7^{-0.0151} X_8^{0.8278} X_{18}^{-0.0168} X_{32}^{0.02} X_{33}^{0.9799} X_{49}^{0.0217}$$

$$\dot{X}_9 = 0.0049 \cdot X_8^{0.7926} X_9^{-0.0007} X_{10}^{0.0207} X_{18}^{0.0208} X_{33}^{0.9799} X_{38}^{0.02} - 3 \cdot 10^{-5} \cdot X_9^{1.036} X_{10}^{-0.0365} X_{18}^{-0.0869} X_{38} X_{49}^{1.0497}$$

$$\dot{X}_{10} = 3 \cdot 10^{-5} \cdot X_9^{1.036} X_{10}^{-0.0365} X_{18}^{-0.0869} X_{38} X_{49}^{1.0497} - 1266.5283 \cdot X_9^{-0.0007} X_{10}^{0.7013} X_{12}^{0.9799} \times X_{13}^{0.2449} X_{18}^{0.0208} X_{37}^{0.9799} X_{38}^{0.02}$$

$$\dot{X}_{11} = 0.8236 \cdot X_6^{0.9648} X_{11}^{-0.0033} X_{34}^{0.9834} X_{35}^{0.0165} - 0.0214 \cdot X_{11}^{0.089} X_{12}^{0.0821} X_{18}^{-0.0868} X_{35}^{0.022} X_{36}^{0.9779} X_{49}^{0.0117}$$

$$\dot{X}_{12} = 9.5864 \times 10^2 \cdot X_{10} X_{13}^{0.25} X_{37} - 0.0209 \cdot X_{11}^{0.064} X_{12}^{0.0839} X_{18}^{-0.0887} X_{36} X_{49}^{0.012}$$

$$\dot{X}_{13} = 0.0202 \cdot X_{11}^{0.032} X_{12}^{0.0419} X_{18}^{-0.0443} X_{36}^{0.5} X_{49}^{0.006} - 1.3359 \cdot X_{10}^{0.3472} X_{12}^{-0.1081} X_{13}^{0.125} X_{37}^{0.5}$$

$$\dot{X}_{14} = 70.1160 \cdot X_{10}^{0.602} X_{12}^{-0.1875} X_{13}^{0.2167} \times X_{14}^{-0.1382} X_{15}^{0.2604} X_{37}^{0.8669} X_{39}^{0.133} - 0.0902 \cdot X_{14}^{0.5118} X_{15}^{-0.1858} X_{39}^{0.1774} X_{43}^{0.8225}$$

$$\dot{X}_{15} = 5.5 \cdot 10^{-5} \cdot X_{14}^{1.9396} X_{15}^{-1.0476} X_{39} - 0.4389 \cdot X_{14}^{-0.7789} X_{15}^{1.4831} X_{39}^{0.75} X_{40}^{0.25}$$

$$\dot{X}_{16} = 0.2526 \cdot X_{14}^{0.2038} X_{43} - 0.7621 \cdot X_{16}^{0.1135} X_{44}$$

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$$\dot{X}_{17} = 0.6302 \cdot X_5^{0.6973} X_7^{-0.0002} X_{17}^{-4.1888} X_{18}^{-0.0036} \times X_{28}^{0.7622} X_{30}^{0.0041} X_{45}^{0.2336} X_{48}^{5.0068} X_{49}^{0.0781} - 1.7294 \cdot X_1^{0.2329} X_3^{0.1322} X_4^{0.0002} X_5^{-0.0006} \times X_6^{0.1385} X_7^{0.01} X_{16}^{-0.0563} X_{17}^{0.5258} X_{21}^{0.2349} X_{25}^{0.0372} \times X_{26}^{0.1879} X_{27}^{0.1221} X_{29}^{0.1979} X_{30}^{0.0094} X_{45}^{0.0778} X_{46}^{0.2546} X_{48}^{0.1166}$$

$$\dot{X}_{18} = 0.1007 \cdot X_5^{0.4601} X_7^{-0.0037} X_8^{0.0086} X_9^{0.2558} X_{10}^{-0.009} X_{11}^{0.0154} \times X_{12}^{0.0203} X_{17}^{-1.629} X_{18}^{-0.0495} X_{28}^{0.5061} X_{32}^{0.0049} X_{36}^{0.2419} \times X_{38}^{0.2469} X_{48}^{1.8734} X_{49}^{0.3193} - 14.3353 \cdot X_7^{0.4330} X_8^{-0.1866} \times X_9^{-1.8 \cdot 10^{-4}} X_{10}^{0.0051} X_{18}^{0.9267} X_{32}^{0.2469} X_{38}^{0.0049} X_{41}^{0.0025} \times X_{42}^{0.2495} X_{47}^{0.4985} X_{49}^{0.2422} \quad (2)$$

This representation may look quite intimidating, but has a number of advantages. First, every parameter has been obtained from published information about the corresponding enzymatic step. As such, it is very easily updated or fine-tuned if improved information becomes available. This has to be seen in contrast to something like a regression model, where typically all parameter values change if data are updated, added, or deleted. Second, each parameter has a unique meaning. For instance, the kinetic order $h_{3,16} = -0.2462$ describes exclusively the effect that citrate (X_{16}) has on the degradation of fructose 6-phosphate (X_3). Its numerical value is negative, indicating inhibition. Third, the particular S-system structure permits numerous algebraic and computational advantages, among which the most important are the explicit representation of the steady state and the analysis of stability and robustness.

3.3 Steady-State Analysis

The steady state of an S-system can be computed with straightforward methods of matrix algebra (Savageau 1969b). In the present case, the steady state coincides with the observed basal state of the citric acid pathway in *A niger*. The steady state of a system is said to be locally stable if the system returns to it after a small perturbation. Local stability is established mathematically by examining the "eigenvalues" of the system (Savageau 1976). If all real parts of the eigenvalues are negative, then the nominal steady state is locally stable. The numerical value of the real part of each eigenvalue indicates the relative time scale of the response involved. The smallest values are related to the slowest processes, while the larger values are associated with the faster processes [e.g., Shiraishi and Savageau (1992)]. Local stability is confirmed by examining the eigenvalues, which all have negative real parts (results not shown). All steady-state and stability analyses are easily executed with the customized freeware PLAS (http://correio.cc.fc.ul.pt/~aenf/plas.html; © António Ferreira).

3.4 Robustness

The explicit nature of the steady state of an S-system permits assessments of the robustness of a biochemical system. Key measures of robustness are "logarithmic gains" and "sensitivities." Logarithmic gains quantify the effects of alterations in independent variables on the steady state. In most good models, changes in an independent variable will not be drastically amplified, that is, the gains will be small in magnitude. Exceptions do exist, for instance, in signal transduction pathways, where small changes in signal are often multiplied several fold. One distinguishes two kinds of logarithmic gains, one addressing a flux and the other a dependent metabolite concentration. Similar to kinetic orders, they are defined as logarithmic derivatives:

Flux logarithmic gain:

$$L(V_j, X_k) = \frac{\partial \ln(V_j)}{\partial \ln(X_k)} = \frac{\partial V_j}{\partial X_k} \frac{X_k}{V_j}$$

Metabolite logarithmic gain:

$$L(X_j, X_k) = \frac{\partial \ln(X_j)}{\partial \ln(X_k)} = \frac{\partial X_j}{\partial X_k} \frac{X_k}{X_j}$$

where X_i stands for a dependent variable (the metabolite concentration) and X_k for an independent variable, which usually codes for an external substrate, enzyme activity, or transport step.

It is not our purpose here to provide a full robustness analysis. Instead, we exemplify the type of analysis and its interpretation with a description of the flux logarithmic gains of the citric acid pathway.

Flux Logarithmic Gains. The flux logarithmic gains are distributed as shown in the three-dimensional plot of Figure 4. They provide quantitative information of the control structure of the system, because high gains indicate that even small changes in the corresponding variable have significant consequences, whereas changes in variables with very small gains are rather irrelevant. A first observation in the present case is that the fluxes most influenced by changes in independent variables are those through the pools of fructose-2,6-bisphosphate (X_4) and isocitrate (X_{15}). In the former case, this behavior is not surprising, because fructose-2,6-bisphosphate is involved in a substrate cycle, and it is known that substrate cycles are rather sensitive to small variations in parameters (Newsholme and Start 1973). The remaining fluxes have low gains with most absolute values below one (97.3% or 543 of 558). The independent variables that have the largest influence on fluxes are those associated with

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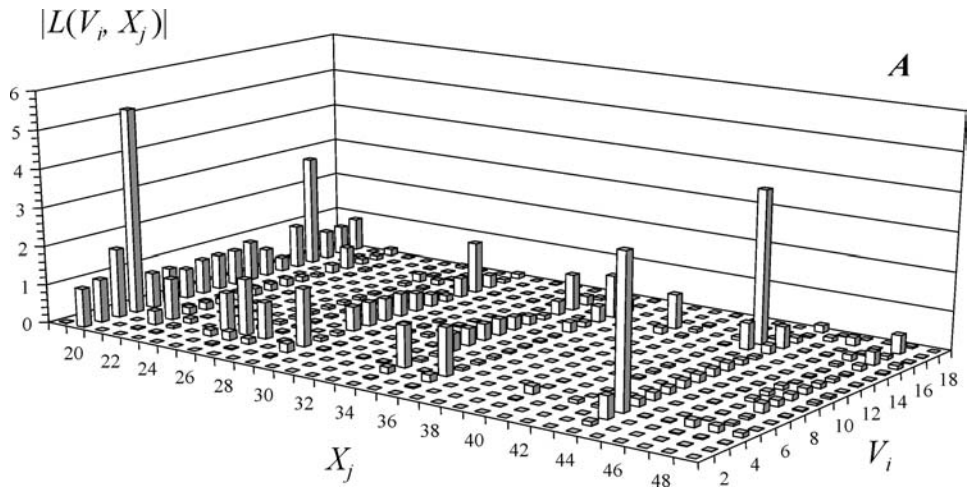

Figure 4 Logarithmic gains of fluxes with respect to changes in independent variables. Fructose 2,6-bisphosphate (X_4) and isocitrate (X_{15}) are affected most strongly.

the transport systems. In particular, the glucose intake system (TRP1, X_{20}) exhibits high gains, which suggests that changes in this carrier would significantly affect the system. Since most gains associated with TRP1 are positive (not shown), increased carrier capacity would lead to increased concentrations and yields.

Several other transport steps are also among the processes that exert the strongest control over the fluxes in the system. These are the pyruvate and the citrate carriers through the mitochondrial membrane (TRP2, X_{34} and TRP4, X_{43}) and the citrate excretion system (TRP5, X_{44}). Similar to the positive gains of the glucose transport system (X_{20}), although of lesser magnitude and affecting a smaller number of fluxes, is the set of gains associated with the mitochondrial pyruvate transport system (X_{34}). The profile in the case of the mitochondrial and cytosolic citrate excretion systems (X_{43} and X_{44}, respectively) is different, indicating mostly negative influence on the fluxes.

The influence of the mitochondrial excretion system (X_{43}) is restricted to the fluxes through the mitochondrial citrate and isocitrate pools (X_{13} and X_{14}, respectively). By contrast, the influence of cytosolic citrate excretion (X_{44}) extends much more widely. In the former case, this means that an increase in the activity of this carrier leaves practically all fluxes unaffected except for those listed above, while in the latter case, most fluxes throughout the system are affected. Other processes with substantial influence on fluxes are the steps catalyzed by glucose 6-phosphate dehydrogenase (X_{22}), cytosolic pyruvate carboxylase (X_{29}), and mitochondrial pyruvate dehydrogenase (X_{36}).

Glucose 6-phosphate dehydrogenase (X_{22}) exerts negative influence (not shown). This is readily explained, because the enzyme is associated with a branch that diverts flux into another pathway. The negative influence is not so evident in the case of pyruvate carboxylase (X_{29}). In this case, the carboxylase feeds the anaplerotic branch of the citric acid cycle and the recycling of oxalacetate through the phosphoenolpyruvate carboxykinase (X_{30}). The influence of pyruvate dehydrogenase is significant.

The remaining variables have minimal effect on the fluxes of the model. Of note is the negligible level of control over phosphofructokinase 1 (PFKI; X_{26}). In spite of the many regulatory features of this enzyme (Arts et al. 1987) and the importance of fructose 2,6-bisphosphate as an activator of PFK1, it seems that the fluxes at the idiophase steady state are by and large insensitive to changes in enzyme activities and effectors.

Rate Constant Sensitivities. A rate constant sensitivity coefficient is defined as the ratio of a relative change in a flux (V_i) or dependent concentration (X_i) to a relative change in a rate constant, α_j (or β_j). These sensitivity coefficients can be determined by differentiation of a steady-state concentration or flux with respect to the parameter (Savageau 1976). The corresponding expressions are:

$$\text{Flux Sensitivity:} \qquad S(V_i, \alpha_j) = \frac{\partial \ln(V_i)}{\partial \ln(\alpha_j)} = \frac{\partial V_i}{\partial \alpha_j}\frac{\alpha_j}{V_i}$$

$$\text{Metabolite Sensitivity:} \qquad S(X_i, \alpha_j) = \frac{\partial \ln(X_i)}{\partial \ln(\alpha_j)} = \frac{\partial X_i}{\partial \alpha_j}\frac{\alpha_j}{X_i}$$

The β rate constants are identical except for a sign change, and hence have the same magnitudes (Savageau et al. 1987).

Most rate constant sensitivities in the citric acid model are less than one in magnitude. This implies that the response to a perturbation in the value of a parameter will be attenuated in most parts of the system. The largest sensitivities occur with respect to the fluxes through fructose 2,6-bisphosphate (X_4), and isocitrate (X_{15}), fructose 6-phosphate (X_3), mitochondrial

citrate, (X_{14}), mitochondrial oxalacetate (X_{10}), and cytosolic citrate (X_{16}) (not shown).

Kinetic Order Sensitivities. A kinetic order sensitivity coefficient is defined as the ratio of a relative change in a flux (V_i) or dependent concentration (X_i) to a relative change in a kinetic order parameter, g_{jk} or h_{jk}. These coefficients are again determined by differentiation of the explicit steady state solution with respect to the parameter in question. The relevant expressions are:

$$\text{Flux Sensitivity:} \qquad S(V_i, g_{jk}) = \frac{\partial (\ln V_i)}{\partial (\ln g_{jk})} = \left(\frac{\partial V_i}{\partial g_{jk}} \frac{g_{jk}}{V_i}\right)$$

Metabolite Sensitivity:

$$S(X_i, g_{jk}) = \frac{\partial (\ln X_i)}{\partial (\ln g_{jk})} = \left(\frac{\partial X_i}{\partial g_{jk}} \frac{g_{jk}}{X_i}\right)$$

The analogous definitions are applicable to the kinetic orders $h_{j,k}$ of the degradation terms.

About 90% of the sensitivities of kinetic orders in the model are less than one in magnitude. The kinetic orders the most pronounced sensitivities are those associated with the transport of glucose, X_1, ($g_{1,20}$, $h_{1,1}$ and $h_{1,21}$); the synthesis and transformation of glucose 6-phosphate, X_2, ($g_{2,1}$, $g_{2,21}$, $h_{2,2}$ and $h_{2,3}$); fructose 6-phosphate, X_3, ($g_{3,2}$ and $g_{3,3}$) and the synthesis and degradation of phosphoenolpyruvate, X_5, ($g_{6,5}$ and $h_{5,5}$).

The results of these types of robustness analyses summarily suggest that the model appears to be reasonable. As to be expected from a natural system, small perturbations in the independent variables or parameter values of the model, as they might occur in response to temperature changes or to errors in transcription or translation, do not cause radical changes in concentrations or fluxes.

3.5 Dynamics

The quality of a model should be examined, both qualitatively and quantitatively, in terms of its dynamic behavior. Numerical solution of the S-system model with PLAS (Ferreira 2000) allows us to predict the responses of the system to perturbations in dependent or independent variables. These responses may be evaluated in terms of deviations from the basal state and in terms of the time it takes to recover from a perturbation.

Figure 5 shows the dynamic response to a two-fold bolus in the dependent variable X_1 (cytosolic glucose). The bolus of glucose is converted in glucose 6-phosphate (X_2), which is partially transformed into fructose 6-phosphate (X_3). Both (X_2) and (X_3) overshoot the basal steady-state concentrations by about 1.5–2.0% over a time period of 15 min, before returning to within 0.5% of the initial steady state. Fructose 6-phosphate is preferentially transformed into fructose 2,6-bisphosphate (X_4), initially (for about 2–4 min) at the expense of the synthesis of phosphoenolpyruvate (X_5), which exhibits small fluctuations around the steady-state concentration (below 1%) before returning to the steady state. From this point on, the perturbation propagates through the anaplerotic oxalacetate synthesis branch and the intramitochondrial reactions, with minimum deviation from the basal steady state (less than 0.5%; results not shown). The transient behavior is characterized by damped oscillations, which are to be expected from a pair of eigenvalues with nonzero imaginary parts that the system has (data not shown). Overall, the time courses show that after 30–40 min all metabolite concentrations have returned to within 1% of the reference steady state, and that only the intermediate concentrations X_2, X_3, X_4, and X_5 deviate by more than 2%. Such a response appears to be biologically reasonable.

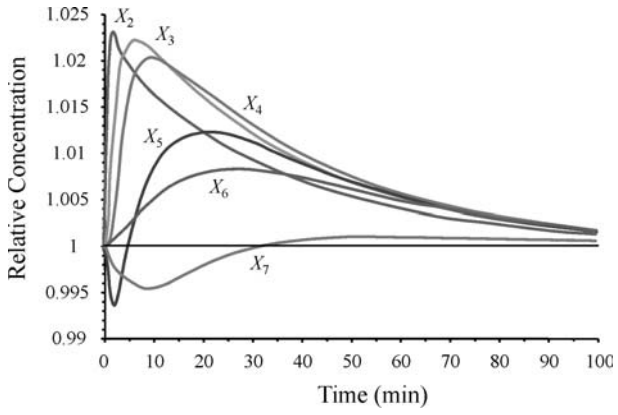

Figure 5 Dynamic system response to a two-fold increase in cytosolic glucose (X_1). At time 0, the cytosolic glucose pool (X_1) was increased to twice the steady state. The six metabolites represented are glucose 6-phosphate (X_2), fructose 6-phosphate (X_3), fructose 2,6-bisphosphate (X_4), phosphoenolpyruvate (X_5), cytosolic pyruvate (X_6) and cytosolic oxalacetate (X_7). The remaining metabolites exhibit negligible deviations form the basal steady-state values (below 0.5%). Each concentration is normalized with respect to its nominal steady-state value.

4 OPTIMIZATION OF *ASPERGILLUS NIGER* METABOLISM DURING CITRIC ACID PRODUCTION

One of the prominent tasks in fermentation technology is the development of strains with increased production performance of a desired metabolite. Traditionally, this task has involved a series of iterations of random mutagenesis followed by selection. While originally very successful, the efficiency of this strategy is beginning to slow down noticeably for many of the well-studied systems. A reason may be that "simple" solutions, requiring alterations in just one or two steps, have been found already, and that any further significant advances would require simultaneous

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changes in several steps. S-system modeling renders it possible to design such multichange strategies for improving yield.

4.1 S-System-Based Optimization Approach

Optimization in biotechnology and fermentation can take many forms [cf. Torres and Voit (2002)]. We concentrate here on the optimization in a steady-state batch culture and illustrate all concepts in the context of citric acid production and excretion. Even this type of optimization is by no means trivial, because numerous enzymatic steps (or genes) could be the targets of manipulation, and alterations guided by intuition are not always successful [e.g., Schaaff et al. (1989)]. Furthermore, the organism might not be able to tolerate drastic changes in some steps or vast increases in some of the metabolite levels, so that any optimization must be constrained. Probably most challenging is the fact that biochemical systems are genuinely nonlinear and that nonlinear optimization problems are notoriously difficult to solve.

The key advantage of formulating the biochemical pathway as an S-system model is the fact that the steady state in this representation is characterized by a system of linear algebraic equations in logarithmic coordinates (Savageau 1969b; 1976). Furthermore, typical optimization objectives can be represented by linear functions in logarithmic coordinates, and the same is true for constraints on fluxes and metabolites, which can be formulated as linear equations or linear inequalities. These crucial features of S-system models have as a consequence the entire problem of optimizing the yield under steady-state conditions becoming a straightforward linear programming task (Voit 1992). This insight has led to numerous applications and to extensions toward optimizing the structure of pathways [e.g., Alvarez-Vasquez et al. (2002), Hatzimanikatis et al. (1996), Petkov and Maranas (1997), Regan et al. (1993), Torres et al. (1996, 1997)]. Upon formulating a biochemical pathway as an S-system, the problem of optimizing a particular flux under typical constraints reads as follows.

4.1.1 Linear Program

(a) Maximize ln(flux)
subject to
(b) Steady-state equations, expressed in logarithms of variables
(c) ln(dependent or independent variable) $\leq$ constant
(d) ln(dependent or independent variable) $\geq$ constant
(e) ln(dependent or independent variable) $=$ constant
(f) ln(dependent or independent variable) unrestricted
(g) ln(flux) $\leq$ constant
(h) ln(flux) $\geq$ constant
(i) ln(flux) $=$ constant
(j) ln(flux) unrestricted

In this formulation, (a) is a typical objective function that is linear in the logarithms of the involved dependent and independent variables; (b) assures that the optimized system is in a steady state, no matter what the altered enzyme concentrations are; (c) and (d) constrain variables to stay within certain limits. (e) forces the variable to be at a given value, whereas (f) is an option that permits any real value for the logarithm of a variable, and thus any positive real value for the variable itself. (g)–(j) are the corresponding constraints on fluxes. Numerous software packages are available for dealing with these types of linear programs. We used the package LINDO PC 5.3 (LINDO Systems INC, Chicago, IL).

4.2 Maximization of Citric Acid Excretion in *Aspergillus niger*

4.2.1 Objective Function

The rate of citric acid excretion is represented in our model by the degradation term V_{16}^- of the 16th differential equation in model [see Eq. (2)]. It has the form $V_{16}^- = 0.7621 \cdot X_{16}^{0.1135} X_{44}$. Taking logarithms transforms the equation into a linear equation of the form $\ln(V_{16}^-) = \ln(0.7621) + 0.1135 \cdot \ln(X_{16}) + \ln(X_{44})$. Since a function and its logarithm assume their maxima for the same values of their arguments, it is legitimate to maximize $\ln(V_{16}^-)$ instead of V_{16}^- itself. Furthermore, the location of the maximum is not affected by the additive term ln(0.7621), so that this term may be ignored during the optimization procedure. The term is therefore simply recorded and only recalled after the optimization, in order to compute the value of the maximized rate of citric acid excretion. Defining $y_{16} = \ln(X_{16})$ and $y_{44} = \ln(X_{44})$, we obtain as the linear objective function the term $0.1135 \cdot y_{16} + y_{44}$. This is the function to be maximized.

4.2.2 Constraints

It is beyond the scope of this review to provide a detailed description of all constraints and their implementation, and we refer the interested reader to the published literature (Alvarez-Vasquez et al. 2000; Torres and Voit 2002; Torres et al. 1996). Nevertheless, it is useful to present issues of optimization constraints at least in concept. Four categories of constraints should be considered.

Steady-State Constraints. These constraints assure that the pathway operates at a steady state, no matter how some of the enzyme activities or transport steps are altered under the optimized regimen. To implement these conditions mathematically, each differential equation is set equal to zero, its degradation term (see Eqs (1) and (2)) is moved to the left-hand side, and both sides are transformed to linearity by taking logarithms.

Constraints on Control Variables. These constraints reflect the range of experimentally feasible alterations of the pathway. Specifically, they limit by how much each

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of the enzyme activities or transport steps is allowed to vary. Recombinant DNA techniques suggest that alterations of enzyme activities may be experimentally implemented within a range between about 0.1–50 times their basal values (Guarante et al. 1980).

Constraints on Metabolites. In order to guarantee a physiologically meaningful solution, it is necessary to limit the ranges of operation for all metabolites. It is unknown and difficult to estimate what magnitudes of deviations from the normal concentrations may be inconsequential. We limit the ranges of all metabolite pools conservatively to 20% about their steady-state levels.

Constraints against Metabolic Burden. Our goal is maximization of productivity and excretion of citric acid, and it is reasonable to expect that this goal can only be attained if the organism is not being altered to a point of compromised viability. It is therefore advantageous to add another type of constraint that is designed to keep the metabolic system within a functional range. This constraint sets an upper limit on the total changes in all altered enzymes combined. One might imagine that a cell could tolerate a 50-fold overexpression in one or two enzymes. However, if the results of an optimization analysis called for 50-fold overexpression of many or all involved enzymes, the cellular metabolism could be overburdened in terms of general functioning, maintenance, and metabolite solubility [e.g., Bentley et al. (1990)]. These reasons of metabolic burden and cellular protein economy suggest that we impose a constraint on the total concentration of all involved enzymes, in addition to the maximally permissible amplification factor for each enzyme.

In the present case we have explored various scenarios. First, we forced the total enzyme amount to remain at the original level, while trying to optimize production through a targeted redirection of fluxes. Secondly, relaxing the constraints against metabolic burden, we optimized the system while allowing the collective enzyme pool to increase by factors of up to 2, 5, or 10 calling it cellular protein economy factor.

4.3 Optimal Solutions

The main results are summarily shown in Table 1. The first column lists all independent variables that are assumed to be available for experimental manipulation. It also includes

Table 1 Normalized optimal enzyme profiles $[(X_i)_{\text{optimum}}/(X_i)_{\text{basal}}]$ and scaled rate of citrate excretion $[SRCE = (V_{16})_{\text{optimum}}/(V_{16})_{\text{basal}}]$ for different values of cellular protein economy. Enzyme activities are given in μM/min

		$(X_i)_{\text{optimal}}/(X_i)_{\text{basal}}$				
		Cellular protein economy factor				
Enzyme	Basal value	0.5	1	2	5	10
X_{20}	0.032	1.5	3	5.6	13	16.5
X_{21}	0.06	1.1	2.2	4.2	9.7	13.3
X_{23}	15.3	0.6	1.6	3.5	9.9	26.1
X_{24}	0.62	0.1	0.1	0.1	0.1	1
X_{25}	0.21	0.1	0.1	0.1	0.1	1
X_{26}	0.075	1.7	4.1	9	25.3	31.8
X_{28}	0.15	4	12.5	27.2	50	50
X_{29}	0.015	1.1	2.7	5.7	15.2	26.7
X_{30}	0.5	0.1	0.1	0.1	0.1	0.1
X_{32}	5.6	0.6	1.5	3.5	10.1	13.6
X_{33}	0.54	1.7	4.3	9.7	28.1	38.1
X_{34}	0.045	1.6	3.9	8.7	25.1	50
X_{36}	0.217	2	4.9	11.2	32.3	42.6
X_{37}	0.007	1.5	3.8	8.7	25.1	37.7
X_{38}	9.4	1.1	2.7	6.2	18	31.5
X_{39}	0.028	0.1	0.1	0.1	0.1	1
X_{42}	0.2	50	50	50	50	10.1
X_{43}	0.009	2	5.1	12.1	37.1	50
X_{44}	0.005	2	5.3	12.4	38	50
X_{45}	2.8	0.1	0.1	0.1	0.1	0.1
X_{46}	5.1	0.1	0.1	0.1	0.4	1
X_{47}	1.0	0.2	0.9	3.1	14.5	50
SRCE	1	2.01	5.17	12.15	37.14	51.05

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the scaled rate of citrate excretion (SRCE), which is the subject of primary interest here. The second column exhibits the basal value of each independent variable and, for easy comparison with other results, the baseline citrate excretion, which is set as "1." The baseline values for enzyme activities were taken from the literature, as discussed in the original descriptions of the optimization task (Alvarez-Vasquez 2000; Alvarez-Vasquez et al. 2000). The body of the table shows the changes in each independent variable and the resulting scaled rate of citrate excretion for any given protein economy factor. Except for the cellular protein economy factor, the remaining constraints were the same in all five optimizations.

The optimal profiles (Table 1) show various interesting results and contain information that will be useful for future optimization purposes. All transport processes, namely, the glucose carrier (X_{20}), the mitochondrial malate transport system (X_{33}), the mitochondrial pyruvate transport system (X_{34}), the mitochondrial citrate carrier (X_{43}), and the cytosolic citrate carrier (X_{44}) must be amplified considerably if a significant increase in the rate of citrate production is to be obtained. The respiratory system (X_{42}) is the process requiring the highest degree of overexpression, in most cases suggesting expression at the allowable upper limit. This result reflects the importance of oxygen and the mechanisms involved in the overflow metabolism of *A. niger*, when the organism operates under conditions of manganese deficiency (Schmidt et al. 1992; Wallrath et al. 1991). Other metabolic steps whose catalytic activity should be increased are the glycolytic steps, and in particular, the reactions catalyzed by hexokinase (X_{21}), phosphofructokinase 1 (X_{26}), and pyruvate kinase (X_{28}). These results are in agreement with the available experimental evidence, which has identified the glycolytic pathway as critical for citric acid synthesis (Schreferl-Kunar et al. 1989; Steinbock et al. 1991). By contrast, the enzymes of the substrate cycle that are associated with the PFKI effector fructose 2,6-bisphosphate, namely fructose biphosphatase (X_{24}) and 6-phosphofructo-2-kinase (X_{25}), actually contribute more strongly to the increase in citric acid production rate, if their activities are reduced to about 10% of their basal values. This indicates that a high rate of fructose 6-phosphate recycling has a negative effect on the rate of citrate synthesis. The situation is similar in the other substrate cycle of the model, the phosphoenolpyruvate carboxykinase (X_{30}) cycle.

Apart from the respiratory system, only two mitochondrial enzyme activities need to be increased, namely pyruvate dehydrogenase (X_{36}) and citrate synthase (X_{37}). Finally, there is a set of processes, outside the abovementioned substrate cycles, whose activities should be decreased. These processes are involved in the transformation of the adenylate phosphate pools and catalyzed by adenylate kinase (X_{45}) and ATPase (X_{46}). Also to be decreased are the activities of NADHase (X_{47}) and aconitase (X_{39}). The latter catalyzes an equilibrium reaction transforming the mitochondrial citrate (X_{14}) into isocitrate (X_{15}). Its activity should be decreased because it negatively affects the rate of citrate excretion. Overall, the enzyme and transport processes whose activities should be increased belong to the direct pathway from external glucose (X_{19}) to citrate (X_{16}). The remaining enzymes change minimally or are even to be reduced in activity.

Examination of the optimal profile without increase in overall metabolic activity (cellular protein economy factor 1) furthermore shows that at least 13 steps need to be upmodulated for attaining significant increases in the rate of citrate efflux. The situation is quite similar for other economy factors, but the profiles of enzyme activities follow the same pattern. When no limits are imposed on the total enzyme concentration (cellular protein economy factor unlimited), the results do not deviate significantly from the solution obtained for economy factor 10 (not shown). The reason is that other constraints become dominant and render the constraint on cellular protein economy mathematically irrelevant.

5 CONCLUSIONS

One of the great challenges of addressing biotechnological problems with mathematical means is the identification of an appropriate modeling structure and of efficient methods that are based on this structure. It has become clear over the past decades, that not all mathematical approaches are equally well suited. Some simplify the underlying processes too much (an example is the use of linear models), while others become mathematically intractable, as soon as the system of interest is of realistic size (an example is the traditional formulation of rate laws as rational functions). While great progress has been made in the past decades, the field of biomathematical model identification is still an open field of research.

It is to be expected that different modeling strategies are optimal in different situations, and that the search of "the one and best" model is futile. Nevertheless, a large body of experience has demonstrated that canonical power-law models have many desirable features and that they may provide a good candidate framework for biotechnological modeling tasks if lacking information in terms of data and the internal logic of a system preclude the development of detailed mechanistic models. With a minimum of information, canonical models can be set up in a symbolic form, and sometimes this symbolic model already yields insights in its design features. The estimation of parameter values is often the step requiring most effort, but more and better data are now being produced than ever before, and methodological experience is accumulating about the identification of relatively large models, especially in pathway analysis and biotechnology.

As an example, this chapter summarized earlier analyses of the citric acid production in the mould *A. niger*. A widely accepted biochemical scheme of the pathway was translated directly into a canonical S-system model, and this model was completely parameterized from data found in the literature. The analysis of stability, gains, sensitivities, and dynamics demonstrated that the resulting S-system model is robust: Small changes in initial values, independent variables, or

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parameters do not alter the steady state much and evoke dynamic responses that appear to be reasonable.

The canonical model can be used for various tasks that are otherwise difficult or impossible to execute. The task we addressed here was the optimization of the pathway with respect to criteria that are not given by nature but defined by human demand. The results of this optimization identified those enzymatic and transport steps that had the highest likelihood of improving citric acid yield. They also suggested that simultaneous alterations in just a few enzymes would have essentially no effect on yield. An intuitive explanation of this result may be that the intense experimental effort in optimizing this system may already have identified a local optimum that cannot be improved by simple alterations. For instance, the fine-tuning of fermentation conditions, including medium composition, cofactors, pH, and temperature, has co-evolved with new, better performing strains, so that the baseline system in our analysis is difficult to improve in a gradual fashion as it results from the traditional approach of random mutagenesis and selection. Instead, novel, improved solutions seem to require "leaps" away from the current state. Of course, such leaps can occur in almost unlimited ways and, without strong guidance, most would result in inferior production or impaired viability. Only a rigorously structured approach can overcome this challenge.

The complexity of biotechnological systems mandates an interdisciplinary approach where modeling and experimentation are intimately intertwined. Such an approach requires biologists to learn the essentials of mathematical modeling techniques, and mathematicians to develop a deeper appreciation for the complexity and intricacy of biological systems, even if they appear to be simple at first.

ACKNOWLEDGEMENT

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28

Fungal Carotenoid Production

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1 INTRODUCTION

The carotenoids are a group of over 600 natural compounds related by their chemical structure and their biosynthesis from a common biosynthetic precursor. They include carotenes, which have 40 carbon atoms and various numbers of hydrogen atoms, and xanthophylls, derived from the carotenes by the introduction of oxygen atoms (Britton et al. 1998; Goodwin 1976; 1980; 1988; Isler 1971; Sandmann and Misawa 2002). The carotenoids are part of a broader group, the terpenoids, that includes such diverse compounds as menthol, steroids, and rubber.

Many of the most attractive colors seen in living organisms, particularly those in the yellow to red range, are due to carotenoids. This makes the carotenoids ideal pigments for food and cosmetics. Lack of the expected pigment would render some foods produced on a large scale unacceptable: this is the case with astaxanthin in salmon, lycopene in tomato paste, β-carotene in butter, and various xanthophylls in eggs. Small molecules derived from the carotenoids contribute an aroma in many of these foods, and in others including red peppers and saffron.

Photosynthetic organisms cannot survive without carotenoids, as these compounds absorb and transfer light energy to the reaction centers and protect them against photosensitizing damage (Edge et al. 1997). Carotenoids are produced by many nonphotosynthetic bacteria and fungi (Armstrong 1997; Sandmann and Misawa 2002), but not by animals, many of which acquire them from their food sources. A well-known function of carotenoids is as precursors of retinol and retinoic acid, which play essential roles as vitamin A and as a cellular regulatory signal, respectively. The main source of retinoids is β-carotene, widespread in nature, but often insufficient in animal diets. Shortage of β-carotene causes severe damage in some human populations, including many cases of blindness. Various carotenoids, independently of whether they serve as provitamin A, are known or suspected to protect against oxidative damage, sun exposure, spontaneous and induced mutation, cancer proliferation, cardiovascular crisis, and degenerative processes.

The market for commercial carotenoids is dominated by the products of chemical synthesis, but the current preference for natural products favors extraction from biological sources. Organisms used for industrial β-carotene production are algae, such as *Dunaliella salina*, or fungi, such as *Blakeslea trispora,* a member of the Zygomycetes in the order Mucorales. The Mucorales *Phycomyces blakesleeanus* and *Mucor circinelloides* are β-carotene producers of potential industrial interest and they are valuable sources of information on the biosynthetic pathway as they are amenable to genetic analysis. The few β-carotene producers outside the Mucorales include *Aspergillus giganteus* (Nowak et al. 1982), *Cercospora nicotianae* (Ehrenshaft and Daub 1994), and *Aschersonia aleyroides* (van Eijk et al. 1979). The main biological source of the carotenoid astaxanthin is the Basidiomycete yeast *Xanthophyllomyces dendrorhous*. Many other fungi produce various carotenoids and deserve attention from industrial microbiologists. For example, torulene and neurosporaxanthin of the ascomycete *Neurospora crassa* are commercially unattractive, but the genetic system of this fungus is a powerful research tool.

Easy cultivation, simple requirements, rapid growth, and high density in culture have favored the use of some fungi in large-scale industrial fermentations for the production of metabolites of applied interest. This chapter reviews the biochemistry and genetics of fungal carotenoid biosynthesis, with emphasis on its regulation and possible applications.

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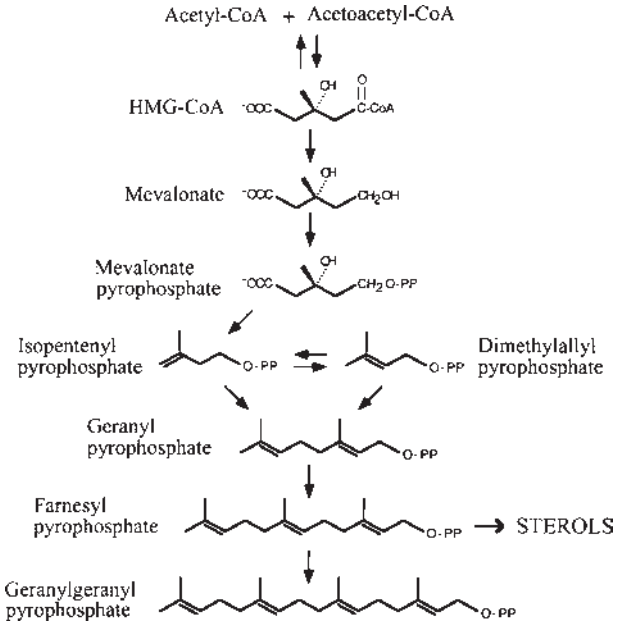

Figure 1 First steps of the terpenoid pathway.

2 BIOSYNTHESIS OF FUNGAL CAROTENOIDS

All terpenoids derive from a five-carbon precursor, isopentenyl pyrophosphate, that may be synthesized by either the mevalonate (Figure 1) or the Rohmer pathway. 3-Hydroxymethylglutaryl coenzyme A (HMG-CoA) and mevalonate are major intermediates of the first and D-1-deoxyxylulose 1-phosphate of the second (Rohmer 1998). The Rohmer pathway does not occur in the fungi. The genes coding for the two first enzymes of the mevalonate pathway, HMG-CoA synthase and HMG-CoA reductase, have been cloned from *Phycomyces* (Ruiz-Albert et al. 2002) and internal sequences of the gene for HMG-CoA reductase from four other Mucorales, are also known including *Blakeslea* (Burmester and Czempinski 1994). The whole sequence of the same gene from *Fusarium fujikuroi* has also been determined (Woitek et al. 1997).

Terpenoid biosynthesis proceeds through the condensation of five-carbon units to form increasingly longer molecules such as geranyl pyrophosphate, farnesyl pyrophosphate (precursor of the sterols), geranylgeranyl pyrophosphate, and further. Phytoene, the first carotene, is produced by the

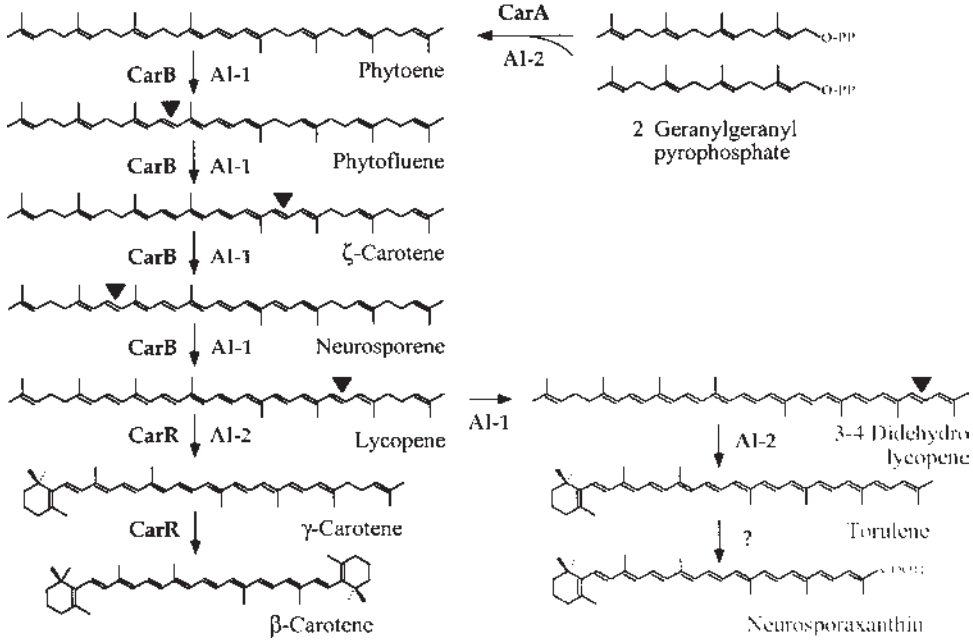

Figure 2 Biosynthetic pathways for β-carotene in the Mucorales and neurosporaxanthin in *Neurospora*. The arrows are labelled with the names of the gene products responsible for the corresponding step (CarA, CarB and CarR in *Phycomyces*, Al-1 and Al-2 in *Neurospora*). CarR and CarA are coded by the bifunctional gene *carRA*. Dehydrogenations are indicated by a grey triangle. Reactions and carotenoids exclusive for *Neurospora* are indicated in grey. The gene responsible for the conversion of torulene to neurosporaxanthin is unknown.

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condensation of two molecules of geranylgeranyl pyrophosphate with the enzyme phytoene synthase. Phytoene is converted to β-carotene via the six steps of the Porter and Lincoln pathway, which include four dehydrogenations and two cyclizations (Figure 2). Phytoene is colorless, but successive dehydrogenations produce molecules with conjugated double bonds that absorb light of progressively longer wavelengths. There are two types of cyclization that give rise to different ring isomers, α and β, depending on the position of a double bond. The name β-carotene denotes the presence of β-rings at the ends of the molecule. Further possible modifications are the introduction of oxygen atoms and oxidative breakage, which gives rise to the smaller derivatives, the apocarotenoids. Thus, neurosporaxanthin is a 35-carbon apocarotenoid with a β-ring at one end and a carbonyl group at the other.

3 BIOSYNTHESIS OF β-CAROTENE BY MUCORALES

Carotene biosynthesis in *Phycomyces* can be interrupted by mutations in one of three genes, *carA*, *carB*, and *carR* (Cerdá-Olmedo 1989; Meissner and Delbrück 1968), which correlate with defects in the three enzymatic functions, phytoene synthase, phytoene dehydrogenase, and lycopene cyclase, respectively. The functional loss of the last enzyme results in red mycelia that contain large amounts of lycopene and loss of the other enzymes results in white mycelia that contain only traces of carotenes (*carA*) or large amounts of phytoene (*carB*).

The genetic analysis of the *car* mutants (Cerdá-Olmedo 1985; De la Guardia et al. 1971; Ootaki et al. 1973; Roncero and Cerdá-Olmedo 1982; Torres-Martínez et al. 1980) led to the identification of two closely linked genes, *carB* and *carRA*. The second is a bifunctional gene with a 5′ domain defined by missense *carR* mutations and a 3′ domain defined by *carA* mutations. Complete loss of function of this gene, for example, by a nonsense mutation in the 5′ domain, gives rise to *carRA* mutants with white mycelia that lack both phytoene synthase and lycopene cyclase. Similar results have been obtained from the mutational analysis of *Mucor circinelloides* (Navarro et al. 1995; Velayos et al. 1997) and *Blakeslea trispora* (Mehta and Cerdá-Olmedo 1995).

The *carB* genes of *Phycomyces* (Ruiz-Hidalgo et al. 1997) and *Mucor* (Velayos et al. 2000a) have been isolated from their homologs in *Neurospora* (Schmidhauser et al. 1990) and *Cercospora nicotianae* (Ehrenshaft and Daub 1994) and the respective *carRA* genes (Arrach et al. 2001; Velayos et al. 2000b) were obtained by chromosome walking, due to their proximity to *carB*, in parallel with the isolation of their homolog in *Xanthophyllomyces* (Verdoes et al. 1999b). In both *Phycomyces* and *Mucor*, genes *carB* and *carRA* are transcribed divergently from a 1-kb regulatory DNA sequence that facilitates their coordinated expression.

All-*trans* β-carotene is the major product of the pathway in *Phycomyces* and *Blakeslea*, but *carB* mutants accumulate 15-*cis* phytoene (Goodwin 1980; Than et al. 1972). The intermediates between phytoene and β-carotene are all-trans isomers, so that there should be an isomerization of phytoene. This may be a consequence of the dehydrogenation of phytoene, because no isomerase activity has been reported and genetic analysis has failed to find isomerase-deficient mutants, which would be white and would form a new complementation group.

The carotenogenic enzymes are partially inhibited by various chemicals, including diphenylamine and cinnamic alcohol for the dehydrogenase (Bejarano and Cerdá-Olmedo 1989) and nicotine and (2-[4-chlorophenylthio]-triethylamine) for the cyclase (Candau et al. 1991). When the pathway is interrupted by mutations or chemical inhibitors, the overall activity increases.

The quantitative determination of intermediates in heterokaryons of color mutants and after the application of chemical inhibitors show that *Phycomyces* has a carotenogenic enzyme aggregate that works as an assembly line and transfers the substrates from one enzyme to another. The aggregate is composed of at least four copies of phytoene dehydrogenase and two copies of lycopene cyclase (Aragón et al. 1976; Candau et al. 1991; De la Guardia et al. 1971) and is probably located in special lipid globules (Riley and Bramley 1982).

The biosyntheses of various other terpenoids share the early enzymatic steps, but occur in different cellular compartments and are independently regulated. This is the case for the biosyntheses of carotenes and sterols in *Phycomyces* (Bejarano and Cerdá-Olmedo 1992) and carotenoids, sterols, and gibberellins in *Fusarium* (Domenech et al. 1996). This was deduced from the comparison of the specific radioactivities of compounds when various strains were grown in the presence of radioactive terpenoid precursors. The separation of such pathways is also supported by the observation that the sterol content is not affected by regulatory mutations or environmental changes that induce large variations in the carotene content.

3.1 Regulation

As it is often the case in the evolution of metabolic pathways, the biosynthesis of carotene is much more conserved than its regulation. The Mucorales provide good examples of this, and the practical consequence is that advances in the construction of overproducing strains in one organism cannot be automatically extended to others.

In *Phycomyces*, β-carotene shortages, produced by genetic or chemical blocks of either the dehydrogenation or the cyclization reactions, lead to the overall activation of the pathway. Thus, in the absence of other activators, the phytoene and lycopene contents of *carB* and *carR* mutants, is about 50 times that of the β-carotene content of the wild type under the same circumstances (Bejarano et al. 1991). This is

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attributed to the regulation of the pathway by its end product (Bejarano et al. 1988). The increase is less pronounced in the *carB* mutants of *Mucor* (Navarro et al. 1995; Velayos et al. 1997) and *carB* and *carR* mutants of *Blakeslea* produce less than twice the amount of the wild type (Mehta and Cerdá-Olmedo 1995).

Many environmental factors influence the carotene content of Mucorales. The effects of media and culture conditions were investigated in *Phycomyces* by Goodwin (1980); Lilly et al. (1960) and in *Blakeslea* by Ciegler (1965), and these studies have provided the background for all later work. Four groups of major agents with different mechanisms of action have been recognized in *Phycomyces*: blue light, β-ring compounds, phenols, and interaction with a strain of opposite mating type. (Bejarano et al. 1988; Govind and Cerdá-Olmedo 1986). The extent of the action of these agents on other Mucorales depends on the species and the strain under investigation.

3.1.1 Regulatory Mutants

In *Phycomyces* it is easy to isolate deeply pigmented mutants that contain up to about one hundred times the β-carotene content of the wild-type. This overproduction is caused by mutations in at least three genes, *carS* (Murillo and Cerdá-Olmedo 1976), *carD* (Salgado et al. 1989), and *carF* (Mehta et al. 1997). It has been proposed that the end-product regulation of the pathway is mediated by an interaction of β-carotene with the CarS and CarA gene products (Bejarano et al. 1988; Murillo and Cerdá-Olmedo 1976).

Lightly pigmented mutants that contain less β-carotene than the wild type are due to mutations in the genes *carI* (Roncero and Cerdá-Olmedo 1982) and *carC* (Revuelta and Eslava 1983). The *carI* mutant and a *carA* allele that were isolated from strains with a reduced response to retinol (Roncero and Cerdá-Olmedo 1982) were deficient in all stimulation processes of carotene biosynthesis. The *carA* domain of gene *carRA* has a dual structural and regulatory function (Arrach et al. 2001), probably through the interaction of its product, phytoene synthase, with the *carI* gene product that modulates its activity.

Carotene-overproducing mutants have been found in *Blakeslea* (Mehta and Cerdá-Olmedo 1995) and *Mucor* (Navarro et al. 1995), but they do not compare to those of *Phycomyces*, and their carotene content is less than five times that of the wild type. Mutants with no carotenes or with less carotene than the wild type have also been found in *Blakeslea* (Mehta and Cerdá-Olmedo 1995) and *Mucor* (Fraser et al. 1996; Navarro et al. 1995; Velayos et al. 1997).

The regulatory gene *crgA* was identified and isolated in *Mucor* because a truncated version of the gene increased the carotene content when introduced into the wild type by transformation (Navarro et al. 2000; 2001). The CrgA gene product is a DNA binding protein whose inactivation stimulates the transcription of the *car* structural gene and gives rise to a β-carotene overproducing phenotype.

3.1.2 Mating Type Stimulation

The increased mycelial color when strains of opposite mating types grow near each other is a well-known feature of the life cycle of the Mucorales (Blakeslee 1904 and Figure 3A). This stimulation of carotenogenesis is not a universal property of the Mucorales; and depending on the species or the strains considered, it may or may not occur, or may occur under different conditions. *Blakeslea trispora* was chosen for the development of industrial procedures because of the increased production of β-carotene in submerged mated cultures, whereas sexual activation in *Phycomyces* is more efficiency on surface cultures (Lilly et al. 1960).

This stimulation does not require physical contact of the mycelia, because it is mediated by an exchange of

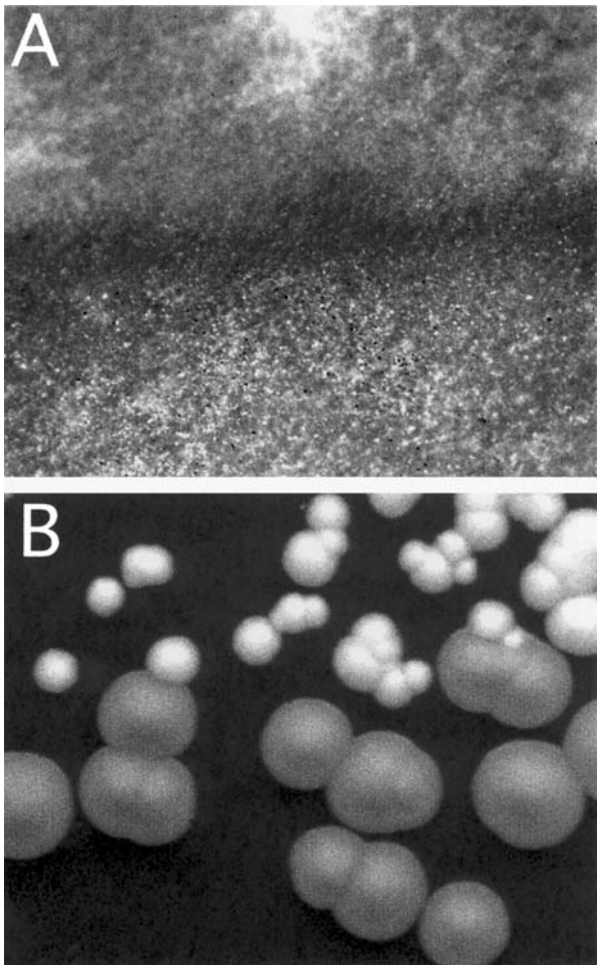

Figure 3 Surface cultures of *Blakeslea* and *Xanthophyllomyces*. A. Encounter between two *Blakeslea* strains of opposite sex. The darker pigmentation in the middle (the natural color is deep yellow) is due to increased β-carotene biosynthesis in interacting mycelia. B. Colonies of wild type (orange in nature) and an albino mutant of *Xanthophyllomyces*.

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pheromones that trigger the mating type differentiation process (Burgeff 1924; Sutter et al. 1974; Sutter 1987). The active compounds are trisporic C and other related trisporoids that stimulate carotenogenesis when added to single cultures (Caglioti et al. 1966).

Trisporoids are derived from β-carotene (Austin et al. 1969) and therefore the structural mutants of the carotene pathway are unable to stimulate opposite mating types, unless they are leaky enough to produce the trisporoids that are needed (Murillo et al. 1978; Sutter 1975). These mutants can be crossed and used in recombination analysis after being placed in heterokaryons with helper nuclei (Roncero and Cerdá-Olmedo 1982).

Single cultures with appropriate genetic combinations provide good alternatives to mated cultures. This is the case of "intersexual" heterokaryons, that is, mycelia that contain nuclei of both mating types (Murillo et al. 1978). Such heterokaryons tend to produce mycelial sectors with asymmetrical mixtures of nuclei. They can be stabilized by "balanced lethals," that is, by placing complementing lethal mutations in both kinds of nuclei of the heterokaryon. If in addition the nuclei of the heterokaryons carry *carS* mutations, the β-carotene content of *Phycomyces* can reach 25 mg/g dry mass under standard laboratory conditions. This is about 500 times the carotene content of the *Phycomyces* wild types. Similar heterokaryons of *Blakeslea* obtained in our laboratory contain up to 35 mg β-carotene per g dry mass.

Similarly effective are intersexual partial diploids of *Phycomyces*, which are at least disomic for the chromosome that carries the mating type markers and heterozygous for them. These diploids appear spontaneously among the progeny of crosses and tend to segregate slowly during vegetative growth through the loss of one or the other chromosome (Mehta and Cerdá-Olmedo 2001).

Intersexual heterokaryons and diploids produce very few spores, but the best nuclei and nuclear combinations can be purified by mechanically breaking the segregating mycelia. This can be achieved by stirring or shaking and the resulting fragments can be plated under conditions that allow them to form separate colonies (Mehta and Cerdá-Olmedo 2001).

3.1.3 Chemical Stimulation

There are no universal mechanisms of chemical activation of carotenoid biosynthesis in the Mucorales in particular or in the Fungi in general, and organisms as closely related as *Phycomyces* and *Blakeslea* can be activated by different compounds. Many chemicals increase the β-carotene content of *Phycomyces* by at least two independent mechanisms (Bejarano et al. 1988). One group of activators, represented by retinol or β-ionone, have in common the presence of a β-ring, and they are thought to compete with β-carotene in binding a regulatory protein. A second group is represented by some phenols, such as veratrol and dimethyl phthalate (Cerdá-Olmedo and Hüttermann 1986) and these are synergic with retinol when present simultaneously in the medium (Bejarano et al. 1988).

Retinol and phenols do not stimulate β-carotene production in *Blakeslea*, but carotenogenesis in this fungus is activated by β-ionone, 2,6,6-trimethyl-1-acetylcyclohexene, isoniazid, and iproniazid (Lampila et al. 1985; Ninet et al. 1969). A separate group of chemical activators in the Mucorales that increase carotenogenesis are the trisporoids that were considered earlier.

3.1.4 Photoregulation

Blue light stimulates carotenogenesis in many fungi (Avalos et al. 1993), including *Phycomyces* (Bergman et al. 1973; Bejarano et al. 1991) and *Mucor* (Navarro et al. 1995; Velayos et al. 1997), but not in *Blakeslea* (Sutter 1970). The photoinduction of carotenogenesis in *Phycomyces* is particularly well known (Bejarano et al. 1991). This response is due at least in part to an increased transcription of the structural genes (Ruiz-Hidalgo et al. 1997; Salgado et al. 1991). Blue light induces the biosynthesis of carotene in *Phycomyces* only during a brief period of the life cycle that coincides with the cessation of mycelial growth and precedes the development of the sporangiophores. The increase in carotene content results from two responses, a weak one with a threshold of only about $10^{-5}\,J\,m^{-2}$ and a much stronger one with a threshold of about $10^{2}\,J\,m^{-2}$ that produces a ten-fold increase in carotene content after illumination with $10^{6}\,J\,m^{-2}$. The action spectra of the two responses are similar but not identical, suggesting differences between the respective photosensory systems.

Some of the components of the regulatory chain responsible of photocarotenogenesis also occur in other light-mediated responses, such as phototropism and photomorphogenesis (Bergman et al. 1973; Cerdá-Olmedo and Corrochano 2001). This is shown by the characterization of mutants that need much higher light stimuli than the wild type to produce the same response. All the *Phycomyces* photoresponses require the action of the genes *madA* and *madB*, defined by mutants with defective phototropism. A search for genetic defects in photomorphogenesis led to the isolation of a *pim* mutant where carotene photoinduction is also defective (Flores et al. 1998). The *picA* and *picB* mutants, however, have defective photocarotenogenesis but are normal in other photoresponses (López-Díaz and Cerdá-Olmedo 1980). The various mutants of gene *carRA* lack photoinduction independently of their carotene content (Bejarano et al. 1991), suggesting that that gene has a regulatory role independent of the enzyme activity. Colored carotenes, including β-carotene, are not needed for photoreception or response, since light increases the phytoene content of *carB* mutants (Bejarano et al. 1991). The same conclusion applies to phototropism, but β-carotene is needed for photomorphogenesis (Corrochano and Cerdá-Olmedo 1990).

Very little is known about the photoregulation of carotene biosynthesis in other Mucorales. In *Mucor circinelloides*, light stimulates the transcription of gene *crgA*, whose product is, paradoxically, a repressor of the structural genes for

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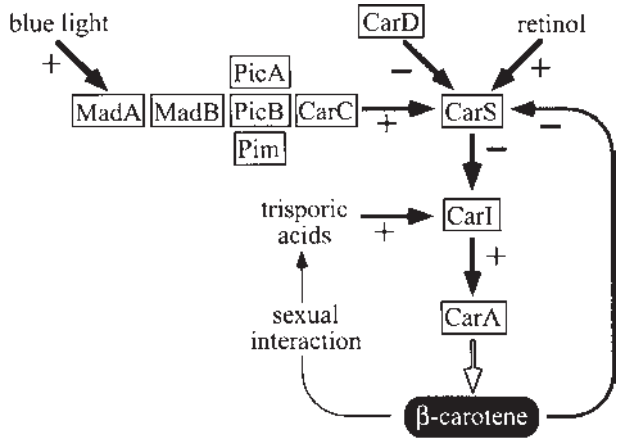

Figure 4 Regulation of carotene biosynthesis in *Phycomyces*. Regulatory gene products are indicated in boxes. The symbols represent stimulatory and inhibitory effects.

carotenogenesis (Navarro et al. 2001). This suggests a complex regulation of plotoregulation in this fungus.

3.1.5 A Regulatory Model for *Phycomyces*

The environmental agents that regulate carotenogenesis in *Phycomyces* and the gene functions determined by genetic analysis of specific mutants can be integrated in a chart (Figure 4) that describes the flow of information from the detection of the environmental agents to the activation of biosynthesis. The scheme is based to a large extent on observations of epistatic interactions and synergisms.

The chart does not establish the molecular mechanisms of action. Increased carotenogenesis *in vivo* is accompanied by an increased carotenogenesis in cell-free extracts (Salgado et al. 1991), but both variables are far from proportional and the latter is usually small in comparison with the former. It is likely that stimulation includes both increased gene expression, such as the transient increase in the transcription of the structural genes that follows illumination (Ruiz-Hidalgo et al. 1997), and activation of the carotenogenic enzyme aggregates.

3.2 Industrial Production

Considerable efforts have been dedicated to establish *Blakeslea* as an industrial organism for the production of β-carotene (Ciegler 1965; Hanson 1967; Ninet and Renaut 1979). β-Carotene is produced in mated cultures of selected strains grown in the presence of chemical activators. Suitable media contain hydrocarbons (kerosene), carbohydrates, and vegetable oils. Production is carried out in two or three culture phases and yields reach 3 g of carotene per liter of medium (Ninet and Renaut 1979). *Blakeslea* is used for industrial carotene production in several countries around the world. The current preference for biological sources against chemical synthesis has increased the interest on *Blakeslea* and on the diversification of its products.

Potential alternatives to *Blakeslea* are *Phycomyces* and *Mucor*. All three Fungi are easy to manage and simple in their growth requirements. The advantages of *Phycomyces* lie in the large amount of information accumulated on several aspects of its biology, including carotenogenesis, and the availability of effective methods to obtain and use mutants, heterokaryons, recombinants, and diploids. The disadvantages are its poor response to mating type stimulation of carotenogenesis in shaken or stirred cultures, and the failure to obtain stable transformants with exogenous DNA. Attempts to optimize industrial carotene production with *Phycomyces* have not been widely published, but growth of appropriate strains on cheap byproducts from biological industries under the usual laboratory conditions can result in 40 g dry biomass per liter with up to 30 mg β-carotene per g biomass (Cerdá-Olmedo 1989). *Mucor circinelloides* produces modest amounts of β-carotene, but the stable transformation of this organism with external DNA offers

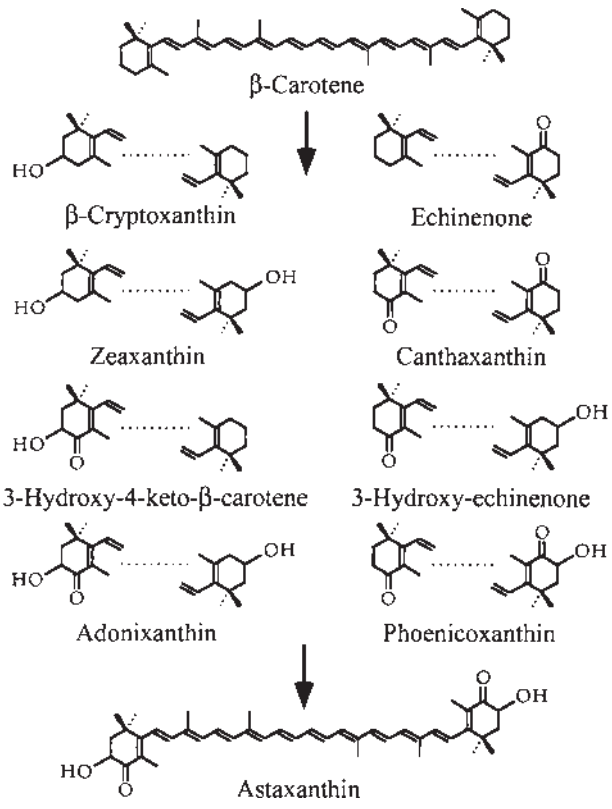

Figure 5 Intermediate xanthophylls resulting from the action of a hydrolase and a ketolase in the astaxanthin biosynthetic pathway. The central segment of the molecule is identical in all intermediates.

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many possibilities for the improvement and diversification of production.

4 BIOSYNTHESIS OF ASTHAXANTHIN BY XANTHOPHYLLOMYCES

Asthaxanthin is a xanthophyll responsible for the pink pigmentation of many animals that cannot synthesize it, including salmonids, boiled crustaceans, and flamingoes. Various microorganisms produce asthaxanthin through the introduction of a keto and a hydroxy group in each of the two rings of β-carotene. There are eight possible carotenoid intermediates between β-carotene and asthaxanthin (Figure 5), but their actual presence depends on the preference of the organism for a certain sequence of reactions.

The basidiomycete yeast *Xanthophyllomyces dendrorhous* (Syn. *Rhodomyces dendrorhous*), formerly *Phaffia rhodozyma*, is a good natural source of astaxanthin (Andrewes et al. 1976; Johnson and Lewis 1979). Isolated originally from exudates found on deciduous trees from cold regions, it can be used as a feed additive to provide the required pigmentation of various salmonids in aquaculture.

The β-carotene biosynthetic pathway of *Xanthophyllomyces* follows the Porter and Lincoln pathway and there are albino mutants that accumulate phytoene or have little or no carotenoids (Girard et al. 1994). Mutants accumulating β-carotene have been reported (Girard et al. 1994), but their enzymatic defect has not been identified. Lycopene-accumulating mutants have not been described.

Genetic analyses of the color mutants by complementation and recombination have been made possible through the use of recombinants (Kucsera et al. 1998), produced through the sexual cycle (Golubev 1995), and heterokaryons (Girard et al. 1994), produced by protoplast fusion. The isolation of mutants and their genetic analysis may be complicated by the ploidy level of wild-type strains. These are unlikely to be simple haploids, as suggested by reports of variability of the ploidy level (Calo-Mata and Johnson 1996), chromosomal length polymorphisms (Cifuentes et al. 1997) and the narrow range of auxotrophic markers that have been isolated (Retamales et al. 1998). The segregation ratios found in tetrads suggest that the zygotes were tetraploid (Kucsera et al. 1998).

Some chemicals work on *Xanthophyllomyces* carotenogenesis much as they do in other fungi (Ducrey Santopietro and Kula 1998). Thus, nicotine inhibits the cyclizations and leads to the accumulation of lycopene. Diphenylamine inhibits not only the conversion of phytoene to lycopene, but also the conversion of β-carotene to astaxanthin. Both of these are oxidative processes.

The genes *crtI*, for phytoene dehydrogenase (Verdoes et al. 1999a), and *crtYB*, for phytoene synthase and lycopene cyclase (Verdoes et al. 1999b), were isolated by heterologous complementation in a *Escherichia coli* strain containing carotenogenic genes from other bacterial sources. The functional characterization of *crtYB* allowed the identification of its homologous genes in other Fungi. The *Xanthophyllomyces* genes for the metabolism of β-carotene to astaxanthin have, however, not been identified, although they may be similar to ketolase and hydroxylase genes from astaxanthin producing bacteria (Fraser et al. 1997) or to genes coding for plant hydroxylases (Bouvier et al. 1998; Sun et al. 1996).

4.1 Regulation

The regulation of astaxanthin biosynthesis in *Xanthophyllomyces* is poorly understood at the genetic level. Because of their potential application, many studies have investigated the identification of overproducing mutants. Such mutants have been isolated by direct visual inspection (Fang and Cheng 1992; Girard et al. 1994), by enrichment methods such as resistance to antimycin (An et al. 1989) and by cell sorting by flow cytometry (An et al. 1991; An 1997). Some of the albino mutants (Girard et al. 1994) may be the result of changes in regulatory, rather than structural genes.

There is no photoinduction of astaxanthin biosynthesis in *Xanthophyllomyces*. On the contrary, bright illumination inhibits growth and decreases the carotenoid content (An and Johnson 1990). Similar amounts of carotenoids are accumulated by the wild type and the β-carotene accumulating mutants and this suggests that feed-back regulation of astaxanthin biosynthesis may not occur (Girard et al. 1994).

Xanthophyllomyces increases its carotenoid content in response to different stress conditions. Moderate increases in astaxanthin biosynthesis are found upon nitrogen or phosphate starvation (Flores-Cotera et al. 2001) or upon addition of ethanol (Gu et al. 1997). Astaxanthin protects the fungus against oxidative stress (Schroeder and Johnson 1993; 1995b) and this protection is extended to fish fed with astaxanthin-rich biomass (Nakano et al. 1999). Active oxygen species, i.e., singlet oxygen, stimulate the pathway (Schroeder and Johnson 1995a), but peroxyl radicals inhibit the introduction of oxygen atoms in the β-rings and the β-carotene concentration in the mycelia increases at the expense of astaxanthin.

4.2 Industrial Production

Current yields of astaxanthin up to about 0.3 mg per g of yeast (Andrewes et al. 1976), obtained from current wild type strains in standard growth conditions, cannot compete with current prices for the chemically synthesized product. Academic laboratories and biotechnology companies have improved both the strains and the culture conditions and in a factorial approach to determine the effects of pH, temperature, inoculum, and carbon and nitrogen supplies (Ramirez et al.) found up to ten-fold variations in carotenoid content. *Xanthophyllomyces* is suitable for both batch and continuous cultures (Acheampong and Martin 1995) and optimal dilution

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rates and pH values have been determined for the latter (Vazquez and Martin 1998).

Synthetic media are not only more expensive, but are often less productive than some complex media, such as hemicellulosic hydrolysates (Parajo et al. 1998), sugar cane juice (Fontana et al. 1996), and peat hydrolysates (Acheampong and Martin 1995; Vazquez and Martin 1998), in which the astaxanthin production can reach to 1.3 mg per g of yeast. The use of overproducing strains also gives increases in yields and up to 8.6 mg per g has been reported (Fang and Cheng 1992).

5 BIOSYNTHESIS OF OTHER XANTHOPHYLLS

Apart from astaxanthin, many other xanthophylls are produced in Fungi, but have not received much attention from biotechnologists. Some of them can be seen as intermediaries in the conversion of β-carotene to astaxanthin (Figure 5). Cantaxanthin, the diketo derivative, is the pigment of the edible mushroom *Cantharellus cynnabarinus* (Haxo 1950) and is also synthesized chemically for the pigmentation of commercial egg yolks. Echinenone, the monoketo derivative, is found in *Peniophora* (Arpin et al. 1966).

Xanthophylls may have undergone no cyclizations. For example, phillipsiaxanthin, found in *Phillipsia carminea*, may be seen as a derivative of lycopene, from which it differs in two dehydrogenations and the introduction of two hydroxy and two keto groups (Arpin and Liaaen Jensen 1967).

Torulene, a carotene obtained from phytoene by five dehydrogenations and a β-cyclization, is present in many fungi, for example *Rhodotorula minuta* (Tada and Shiroishi 1982) and *Verticillium agaricinum* (Valadon and Mummery 1973). Oxidation of its linear end to a carboxyl group gives rise to torularhodin, found in *Rhodotorula* and *Rhodosporidium* (Kockova-Kratochvilova and Bystricky 1974). An oxidative break can remove five carbons and leave a carboxyl group at the linear end, forming neurosporaxanthin (Figure 2). This xanthophyll is of no commercial interest, and is the end product of the carotenoid pathway in fungi that have been studied for various reasons, such as *Neurospora crassa* (Aasen and Liaaen-Jensen 1965), *Fusarium fujikuroi* (Avalos and Cerdá-Olmedo 1986; 1987), and *Verticillium albo-atrum* (Valadon and Heale 1965). The extensive genetic and biochemical techniques developed in *Neurospora* produce results that can serve as a guide in applied research with other organisms.

The albino mutants of *Neurospora* have been used for over half a century as markers for genetic analyses. They belong in three noncontiguous genes, *al-1* through *al-3*. The conversion of farnesyl pyrophosphate to geranylgeranyl pyrophosphate is catalyzed by the product of gene *al-3* (Nelson et al. 1989). *Fusarium* has two genes responsible for this conversion, one of which is used in the production of carotenoids (Mende et al. 1997) and another that is involved in the production of gibberellins (Tudzynski and Hölter 1998). The *Neurospora* gene *al-1* codes for phytoene dehydrogenase, the enzyme that catalyzes the five dehydrogenations that are needed to produce torulene and neurosporaxanthin (Hausmann and Sandmann 2000). This was the first specific gene for carotenoid biosynthesis cloned in fungi (Schmidhauser et al. 1990), as a consequence of its close linkage to another gene that was being investigated. Gene *al-2*, coding for phytoene synthase, was cloned by direct complementation of *al-2* mutants (Schmidhauser et al. 1994). These *Neurospora* genes have helped considerably in the isolation of homologous genes from other fungi.

Neurospora mutants defective in the β-cyclase are reddish and inconspicuous in the wild-type background and have only been isolated recently. They result from changes in the 5′-region of gene *al-2* (Arrach et al. 2002), which is thus similar to the genes *crtYB* of *Xanthophyllomyces* (Verdoes et al. 1999b) and *carRA* of *Phycomyces* (Arrach et al. 2001). The gene responsible for the oxidative break of torulene to neurosporaxanthin has not been identified.

The *Fusarium* genes *carB* and *carRA* (Fernández-Martín et al. 2000; Linnemannstöns et al. 2003) are similar to their namesakes from *Phycomyces*. In contrast to *Neurospora*, the *Fusarium* genes are contiguous, but they are transcribed in the same direction, and not divergently from a common regulatory DNA segment, as in *Phycomyces*.

6 LYCOPENE PRODUCTION

Lycopene is gaining attention in the carotenoid market because of its health-promoting properties (Giovannucci 1999). Lycopene-rich drinks are becoming widespread and there is a trend towards the use of lycopene as a food additive. The industrial production of lycopene has become an attractive goal for fungal biotechnologists.

Any organism used for β-carotene or astaxanthin production is a potential source of lycopene following inactivation of the cyclase. This can be achieved, as described previously, either chemically, for example, with nicotine, or genetically, with β-cyclase-deficient mutants. The structural mutations should be associated with regulatory mutations for carotene overproduction. In order to benefit from mating type stimulation in the Mucorales, any mutants must be leaky and produce some β-carotene. In the case of *Phycomyces*, the β-carotene shortage that follows the inhibition of the cyclase results in a strong activation of the pathway and a large accumulation of lycopene.

Under usual laboratory conditions, the lycopene content reaches 15 mg per g dry mass for both *Phycomyces* (Murillo et al. 1978) and *Blakeslea*. These values can certainly be increased by the application of already available knowledge of optimal growth conditions and genetic effects.

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7 HETEROLOGOUS BIOSYNTHESIS

The heterologous expression of structural genes for carotenoid production may convert organisms unable to produce carotenoids into good sources of these compounds. Thus, *E. coli* was converted into a carotenogenic organism that has produced a wealth of information on the pathway. *Saccharomyces* and *Candida* have become promising candidates for industrial purposes. These yeasts accumulate large amounts of ergosterol and have therefore a very active terpenoid pathway that could provide the precursors for carotenoid biosynthesis. In this way, *Saccharomyces* transformants that contain a plasmid with the genes for geranylgeranyl pyrophosphate synthase, phytoene synthase, and phytoene dehydrogenase from the bacterium *Erwinia uredovora* under different yeast promoters are reddish and accumulate 0.1 mg lycopene per g dry mass (Yamano et al. 1994). The additional insertion of the gene for lycopene cyclase results in the production of β-carotene instead of lycopene.

Candida utilis is another suitable candidate because of its use in large scale protein production processes. The same genes from *Erwinia* have been modified to fit *Candida* codon usage and placed under strong *Candida* promoters. The resulting transformants accumulated about 1 mg lycopene per g dry mass. Addition of a modified gene for lycopene cyclase led to the accumulation of about 0.4 mg β-carotene per g dry mass and further addition of modified genes for β-carotene ketolase and β-carotene hydroxylase led to a similar concentration of astaxanthin (Miura et al. 1998).

Carotenoid production by *Candida* can be improved through the modification of other metabolic steps. A four-fold increase in carotenoid production was obtained (Shimada et al. 1998) when a truncated gene for HMG-CoA reductase that lacked the amino domain required for its degradation (Hampton et al. 1996) was overexpressed in a lycopene-producing strain. The increase was doubled if the recipient strain had only one of the two genes for squalene synthase, the first step in sterol biosynthesis.

Future heterologous gene expression may convert *Blakeslea* and *Xanthophyllomyces* into sources of novel and valuable carotenoids, profiting from the previous improvements in the yields.

8 CONCLUSION

The increasing demand for natural carotenoids is a strong enticement for biotechnologists. *Blakeslea* and *Xanthophyllomyces* are potential industrial sources of β-carotene and astaxanthin, respectively, that could compete with the chemical synthesis of the same compounds, but both organisms have drawbacks. The techniques available for research in *Blakeslea* are relatively primitive, but useful information may be derived from the genetics of *Phycomyces* and the molecular biology of *Mucor*, both close relatives of *Blakeslea*. Both genetic and molecular improvement are possible with *Xanthophyllomyces*, but the organism's slow growth and low optimal temperature limits its attraction for industrial purposes. Large increases in carotenoid production have been obtained from the optimization of the fermentation conditions and strain improvement by classical genetics and further improvements are expected. An alternative to the industrial improvement of carotenoid-producing organisms is the introduction of genes for carotenoid biosynthesis in industrial organisms such as *Candida* and *Saccharomyces*.

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Fungal Terpenoid Antibiotics and Enzyme Inhibitors

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1 INTRODUCTION

Microbial metabolites and their derivatives hold an important position in the development of medicinal drugs. Research on fungal metabolites as medicinal candidates was initiated when Fleming discovered penicillin in the culture of a filamentous fungus identified as *Penicillium notatum*. This was followed by Brotz who discovered cephalosporin in the culture of another filamentous fungus, *Cephalosporium acremonium* (now renamed *Acremonium chrysogenum*). Nowadays, about 30 penicillin derivatives and 49 cephalosporin derivatives are used in clinics in Japan. Although fungal metabolites had been the major target for screening of antimicrobial agents, they were overtaken by metabolites of actinomycetes after Waksman discovered streptomycin in 1945. This might be partly due to the fact that fungi frequently produce mycotoxins with potent cytotoxicity to humans and animals, such as aflatoxins from *Aspergillus flavus*, known to induce chronic hepatotoxicity ultimately leading to a malignant tumor.

However, the trend has changed in recent years, and fungal metabolites have again attracted the attention of pharmaceutical researchers. Statistical figures obtained from the search of literature and patents reveals the important contribution of fungal metabolites as a source of bioactive agents. The percentages of fungal and actinomycete metabolites reported in the Journal of Antibiotics (JA) were 13 and 66% (1983), 10 and 79% (1985), 16 and 74% (1990), 38 and 53% (1994), and 47 and 44% (2000), respectively, while the percentages of bacterial metabolites remained around 8% except for 21% in 1983. A similar tendency was observed in the metabolites claimed in Japanese patent applications. The number of patents of fungal products increased from 11% (1983) to 21% (1990) and to 36% (2000), while that of actinomycetes decreased from 74% (1983) to 66% (1990) and 48% (2000) (Miyadoh 1995). According to Tanaka and Omura, 43% of 8,000 new microbial metabolites reported were discovered by Japanese researchers. Therefore, the screening activity described earlier may represent the activity worldwide.

Abundance of the secondary metabolites in fungi and actinomycetes, as compared with bacteria and yeast, may be associated with the poor nutritional environment where they live. Under poor conditions, it may be necessary for microorganisms to utilize nutrients available for metabolic cycles as much as possible, which may result in production of a variety of secondary metabolites, as seen in lichens. It was suggested that maximum production of secondary metabolites may be achieved by using nutritionally depleted substrates (Aldred et al. 1999).

Regarding antibiotic screening, a survey of JA showed that the number of antifungal metabolites increased markedly, antitumor metabolites moderately, but that antibacterial metabolites had decreased in the last ten years (Yagisawa 2000). However, the most marked increase was seen in the nonantibiotic bioactive metabolites. In this nonantibiotic area, the value of fungal metabolites has become better recognized, since eukaryotic fungal metabolites were expected to be more applicable to the metabolism of mammalian cells than metabolites of the prokaryotic actinomycetes and bacteria. Novel fungal products reported in JA increased dramatically in the last 10 years owing to the popular screening of the cholesterol biosynthesis inhibitors, about 93% being of fungal origin (Yagisawa 2000).

Cultures of microorganisms usually contain a complicated mixture of a variety of low and high molecular-weight compounds. Direct pharmacological screening of an active compound from such a complicated mixture would be

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hampered by the fact that its pharmacological action, e.g., such as central nervous stimulation/or sedation is readily modified, masked, or disturbed with by coexisting components. On the other hand, the enzyme inhibitory activity could be detected reproducibly in the fermentation broth even when an active compound is present in a low amount, as could the antibiotic activity. Coupled with the advancement of biotechnology and introduction of the high-throughout screening with an automated biological assay using robots, the enzyme inhibitors are detected easily and efficiently. Moreover, screening of these molecules that interact with mammalian receptors or signal transduction pathways also becomes possible. Statistical figures in JA showed that the number of bioactive metabolites excluding antibiotics increased from 0 (0%, 1958–1967) to 55 (4%, 1968–1977), 148 (12%, 1978–1987), and 603 (30%, 1988–1997) (Yagisawa 2000).

The terpenoids constitute one of the largest groups of naturally occurring compounds in the plant, animal, and protista kingdoms, being characterized by their great diversity of chemical structure. Secondary terpenoid metabolites are most abundant in plants, and various terpenoids have been developed as important medicinal drugs, among which are reserpine as a hypotensive and sedative agent, vincristine, vinblastine, and taxol as antitumor agents, artemicin as an antimalarial agent, and geranylester of farnesylacetic acid (gefarnate), plaunotol and teprenone as antiulcer agents. In contrast, fewer fungal terpenoids have been developed in the medical field, and in the list of bioactive terpenoids published in 1969 (Martin-Smith and Sneader 1969), an antibacterial agent, fusidic acid (Figure 4) was the only compound of fungal origin. Thereafter, siccanin, a triprenylphenol (Figure 4) was successfully developed in Japan as an antidermatophytic agent (Bellotti and Riviera 1985). However, a variety of volatile monoterpenes and sesquiterpenes are known to be produced by fungi, especially by mushrooms, and therefore there remained a high possibility of discovery of a greater number of bioactive terpenoids from fungi. Indeed, as the screening target was expanded from the antibiotic activity to a wide range of pharmacological activities, discovery of bioactive terpenoids, particularly that of enzyme inhibitors active against the isoprenoid pathways, was markedly increased.

As the clinical utility of the compactin-related 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors became apparent, many pharmaceutical industries interested in the development of cholesterol lowering drugs began to search for new targets of cholesterol biosynthesis inhibition other than reductase. The first alternative target was directed to two enzymes, thiolase and HGM-CoA synthase, both of which catalyze the initial steps of isoprenoid synthesis. Then, the interest of researchers was shifted to a search for inhibitors of squalene synthase (SQS) and some other enzyme steps involved midway along the sterol synthetic pathway. Finally, more attention was directed to the cholesterol metabolism, the details of which were elucidated by the extensive use of HMG-CoA reductase, supported by the advancement of cellular biochemistry. Through these screening processes, a large number of terpenoids, some of which were substrate analogs of enzymatic reactions, have been discovered.

This review summarizes the terpenoid antibiotics and enzyme inhibitors of fungal origin, most of them being reported in the last ten years. Because of space limitation, an exhaustive survey of the extensive literature on the fungal terpenoids is difficult to include here, and only representative terpenoids and other metabolites structurally mimicking either to terpenoid or substrate of enzymes involved in the isoprenoid pathways were chosen as topics. In the case where a large number of homologous or analogous metabolites were isolated from a single fungal culture, the one with highest biopotency or a major metabolite is discussed. The publications cited are confined to the biological reports, and those concerning structure determination and synthesis of the active metabolites are cited only when considered necessary. A minimum of the literature on mechanism of enzymatic action or the genetic designations of the structural genes is also mentioned.

2 ISOPRENOID PATHWAYS

The pathways for isoprenoid biosynthesis are shown in Figures 1 and 2. Since a detailed review of biosynthetic pathways has been made (Dewick 1998), only a brief summary is given here. There are two pathways to isopentenylpyrophosphate (IPP), which is the fundamental building stone to construct higher isoprenoids. Starting from acetyl coenzyme A (CoA), dimerization by acetoacetyl-CoA synthase, followed by the action of HMG-CoA synthase, forms HMG-CoA. This is converted to mevalonic acid (MVA) by the action of HMG-CoA reductase. Mevalonic acid is converted to IPP via three steps.

The nonmevalonate pathway starts with pyruvate and glyceraldehyde-3-phosphate, which is derived from a sugar. Condensation of two precursors by deoxyxylose-5-phosphate (DXP) synthase yields DXP, which is isomerized to 2-*C*-methyl-D-erythritol-4-phosphate (MEP) by the action of DXP reductoisomerase. Three steps of enzymatic reactions lead to 2-*C*-methyl-D-erythritol-2,4-cyclopyrophosphate (MECPP), which is converted by unknown multiple steps to IPP. All animal and fungal cells take the mevalonate route to IPP, while part of the plant, bacteria, actinomycete, and protozoa take the nonmevalonate route. This review concerns the mevalonate route, but the nonmevalonate route is useful when screened for anti-Gram-negative bacterial and antimalarial agents since this route does not exist in humans (Rohmer 1998). Indeed, fosmidomycin, a metabolite of actinomycete, inhibiting 1-deoxy-D-xylulose 5-phosphate reducto-isomerase with IC50 of 8.2 nM (Kuzuyama et al. 1998), was shown to be effective to malarial infection (Jomaa et al. 1999).

Head to tail dimerization of IPP to DMAPP gives geranylpyrophosphate, from which a variety of monoterpenes

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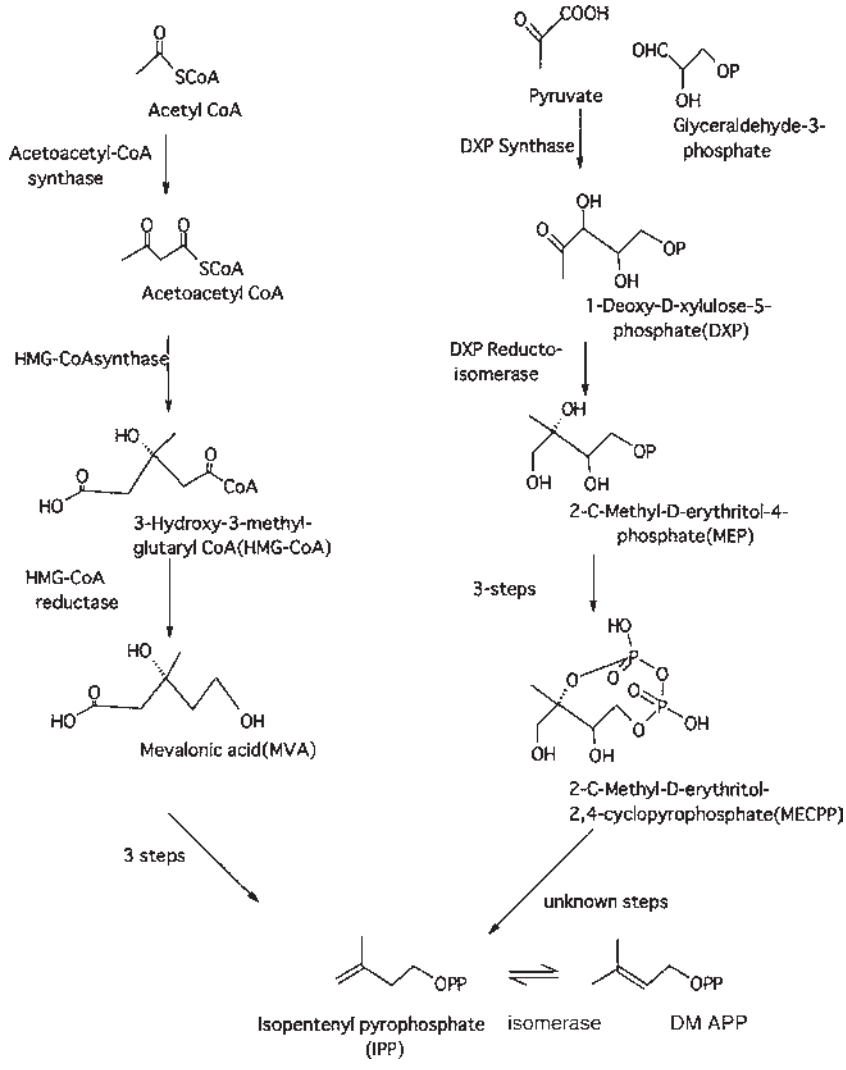

Figure 1 Mevalonate and nonmevalonate pathways.

(C10 terpenoid) are formed. Addition of one more IPP to a geranylpyrophosphate gives farnesylpyrophosphate (FPP), from which sesquiterpenes (C15 terpenoids) are derived. Direct farnesylation of proteins with protein farnesyltransferase (PFT) modifies the nature of proteins, in which Ras protein farnesylation is noted with respect to tumor progression. Addition of one more isopentenyl group to FPP affords geranylgeranylpyrophosphate (GGPP), from which various diterpenes (C20 terpenoids) are produced. Direct geranylgeranylation of various proteins occurs by protein geranylgeranyltransferases to regulate cell proliferation, differentiation, and aptotosis (Tatsuno et al. 1997; Vogt et al. 1996).

Formation of squalene from two molecules of FPP catalyzed by SQS proceeds in two steps: in the first step, head to head condensation of two FPP molecules leads to formation of a stable intermediate, presqualene-pyrophophate (PSPP) containing cyclopropylcarbinyl pyrophosphate, and in the second step, PSPP goes through a loss of inorganic pyrophosphate and a reductive rearrangement to produce squalene (C30 terpenoid). Squalene is converted to squalene 2,3-epoxide by squalene epoxidase. During cyclization of squalene 2,3-epoxide, phytosterols of plant origin are diverted from fungal and animal sterols. Fungal and animal sterols are further divided after lanosterol, the fungal to ergosterol, and the animal to cholesterol in multiple steps.

Figure 3 shows part of the metabolism of cholesterol in humans. Cells in the human body acquire the cholesterol needed for membrane synthesis by means of cell surface receptors (LDL receptors). Binding of low-density lipoprotein (LDL) to its receptor leads to cellular uptake by endocytosis or selective transfer, providing the cell with its cholesterol.

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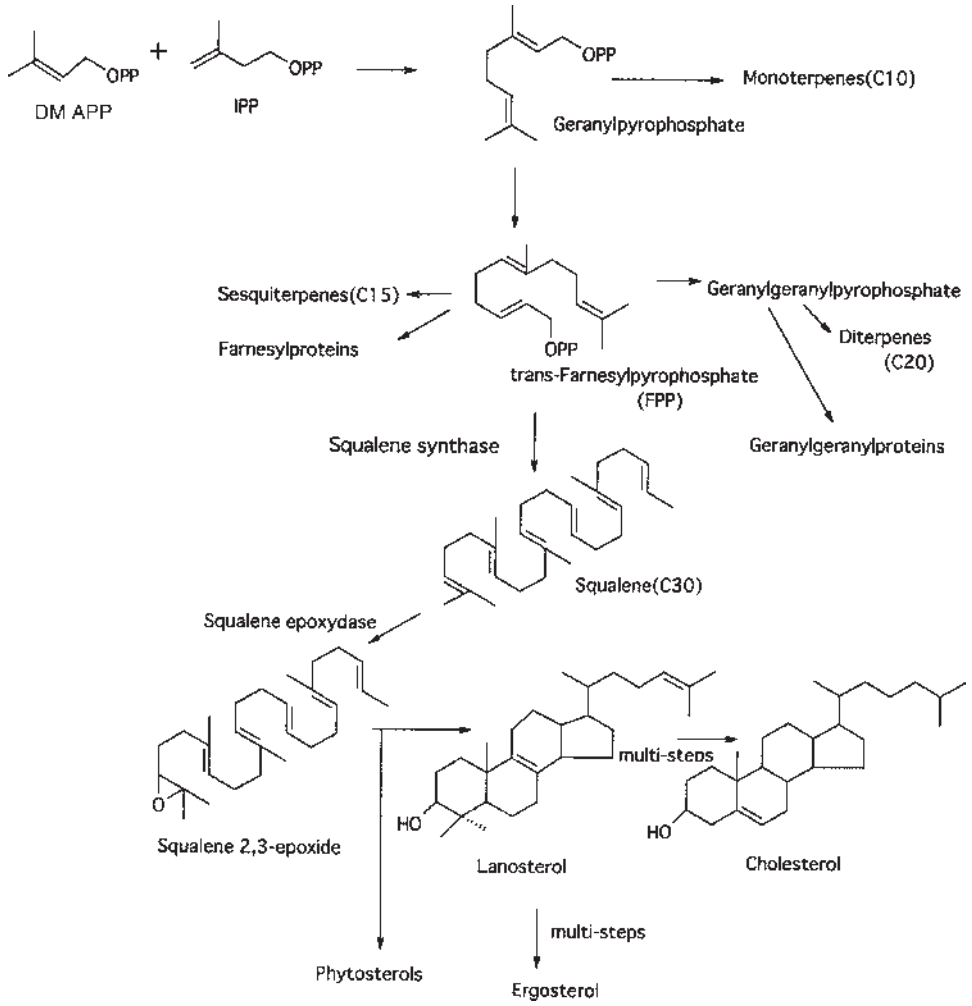

Figure 2 Biosynthesis of terpenoids.

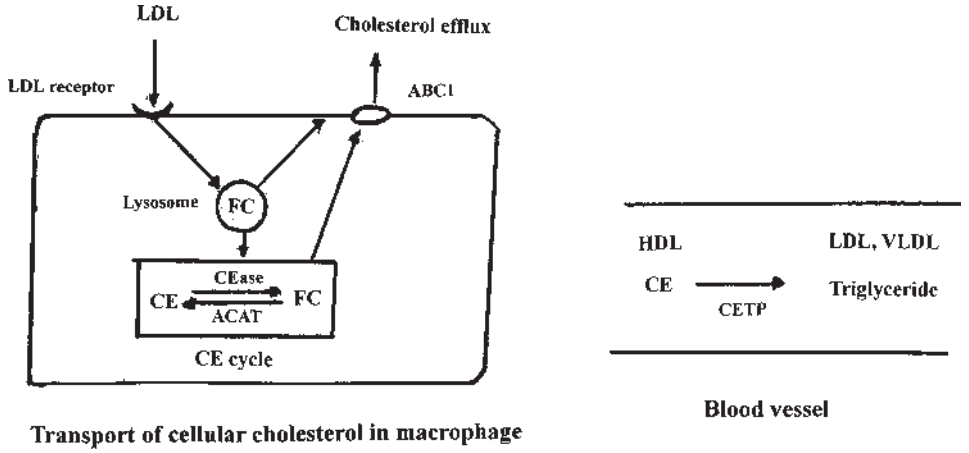

Figure 3 Metabolism of cholesterol in humans. FC, free cholesterol; CE, cholesteryl ester; CEase, neutral cholesteryl ester hydrase; ACAT, acylCoA:cholesterol acyltransferase; ABC1, ATP-binding casette transporter A1; CETP, cholesteryl ester transfer protein.

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Free cholesterol is liberated within a lysozome, and partly binds to the cell membrane as a constituent. On the other hand, to avoid overaccumulation, the supplied cholesterol also suppresses the synthesis of LDL receptors and enzymes of cholesterol synthesis by feedback repression. Excess cholesterol is stored in the form of cholesteryl ester in small vacuoles by the action of acyl CoA: cholesterol acyltransferase (ACAT). When necessary, free cholestereol is produced by cholesterol esterase (CEase). Finally, free cholesterol is excreted either via the active/ABC1 mediated pathway or via the passive/diffusion pathway outside a cell (cholesterol efflux).

There is another metabolic pathway of cholesterol in blood vessels. Cholesteryl ester in high-density lipoprotein (HDL) is transported to LDL and VLDL by exchanging with triglyceride through the action of cholesterol ester transferase protein (CETP). The cholesterol transported to LDL and VLDL is again taken up by the liver through the LDL receptors.

3 FUNGAL TERPENOID ANTIBIOTICS

The chemical structures of terpenoid antibiotics described below are shown in Figure 4.

3.1 Antifungal Terpenoids

Opportunistic fungal infections are increasingly common in severely immunocompromised patients especially following cancer chemotherapy, bone marrow or organ transplantation and HIV infection. The prognosis for these patients is typically very poor. A study of the present antifungal drugs indicates that they have drawbacks such as resistance and toxicity. Consequently, the search for novel antifungal compounds displaying new modes of action and improved pharmacological characteristics has intensified across the pharmaceutical industry.

Over the last ten years, a couple of companies have shown considerable interest in a group of sordarins. Sordarin, which is a tricyclic diterpene glycoside, was first isolated from a fungus by Hauer and Sigg (1971) but was not studied further, probably because of its modest antifungal activity. More recently, new members belonging to the sordarin family were discovered. Zofimarin produced by *Zofiella marina* was discovered by the Sankyo research group in 1987 (Ogita et al. 1987). It showed potent antifungal activity. SCH57404 possessing the rare tricyclic sugar moiety was isolated from an unidentified fungus by the Schering Plough research group in 1995 (Coval et al. 1995) and showed weak anti-candida activity with a narrow spectrum. The same compound under the name of xylarin was isolated from a wood-inhabiting *Xylaria* sp. (Ascomycete) (Schneider et al. 1995). BE-31405, which is remarkably similar to SCH57404, was isolated from the culture of a soil fungus, *Penicillium miniolateum* by the Banyu Seiyaku research group in 1998 (Okada et al. 1998). It showed a potent activity against *Candida* and *Cryptococcus* species, and inhibited the protein synthesis of *Candida albicans*, but not of mammalian cells.

In 1998, GR135402 was reported by the Glaxo Wellcome research group, having been isolated from the culture of *Graphium putredinis* (Kinsman et al. 1998). GR135402, differing from sordarin only in the presence of a 2-methylhex-2,4-dienoate side chain showed highly potent activity against several important fungal pathogens: *C. albicans*, *Cryptococcus neoformans*, *Pneumocystis carinii*, and dimorphic endemic fungi.GR135402 is a potent inhibitor of the protein synthesis elongation cycle in *C. albicans* (IC50, 0.03 μg/ml), but is not in rabbit reticulocytes, indicating a high level of selectivity. Indeed, GR135402 and its minor metabolites were discovered during a screening program for inhibitors of fungal protein synthesis. Structure–activity study revealed that the nature of the 3′-O-acyl side chain in the sugar moiety had a profound effect on the potency of the compounds. The important role of the 3′-O-acyl group in conferring potent antifungal activity is demonstrated by the low potency of sordarin without 3′-substituent (Kennedy et al. 1998).

An extensive derivatization of the sordarin antibiotics was carried out by the Glaxo SmithKline research group. Biotransformation of sordarin employing *Streptomyces avermitilis* gave 4′-O-demethylsordarin, which led to the synthesis of GM237354 (Herreros et al. 1998), subsequently progressed to preclinical development by GlaxoSmithKline. The MIC90s of GM237354 at which 90% of the isolates were inhibited were 0.015 μg/ml for *C. albicans*, 0.12 for *C. tropicalis*, 0.03 for *C. kefyr*, and 0.25 for *C. neoformans*. The 50% inhibition of *P. carinii* was <0.008 μg/ml. It was active against some filamentous fungi, but weak against *C. krusei*, *Aspergillus fumigatus*, and dermatophytes. GM237354 exhibited therapeutic efficacy in murine models of systemic candidiasis (Aviles et al. 2000), pneumocyctosis, coccidioidomycosis, and histoplasmosis by subcutaneous dosing.

A further evolution of this class of compounds has led to a new family (GW471558 etc) called azasordarins, in which the sugar moiety is replaced with N-substituted morpholines (Herreros et al. 2001). GW471558 showed potent antifungal activity *in vitro* and *in vivo* with similar spectrum to that of GM237354 (Martinez et al. 2001).

Several antifungal metabolites with steroidlike structure were isolated from fungi. A25822 A and B discovered by the Eli Lilly research group are azasteroids produced by *Geotrichum* sp. (Gordee and Butler 1975). A25822B was primarily active against *C. albicans* and *Trichophyton mentagrophytes* as well as *C. neoformans*, *Histoplasma*, etc. A25822 inhibited delta14-sterol reductase (Bottema and Parks 1978), and was effective *in vivo* by parenteral and oral administration. Recently, A25822B (UCA1064-A) was rediscovered as an antitumor antibiotic from *Wallemia sebi*, which was isolated from dried potato (Takahashi et al. 1993).

Mer-NF8054 A and X were isolated from various species of *Aspergillus* collected from soil by the Mercian research group (Sakai et al. 1994). The antifungal activity was very

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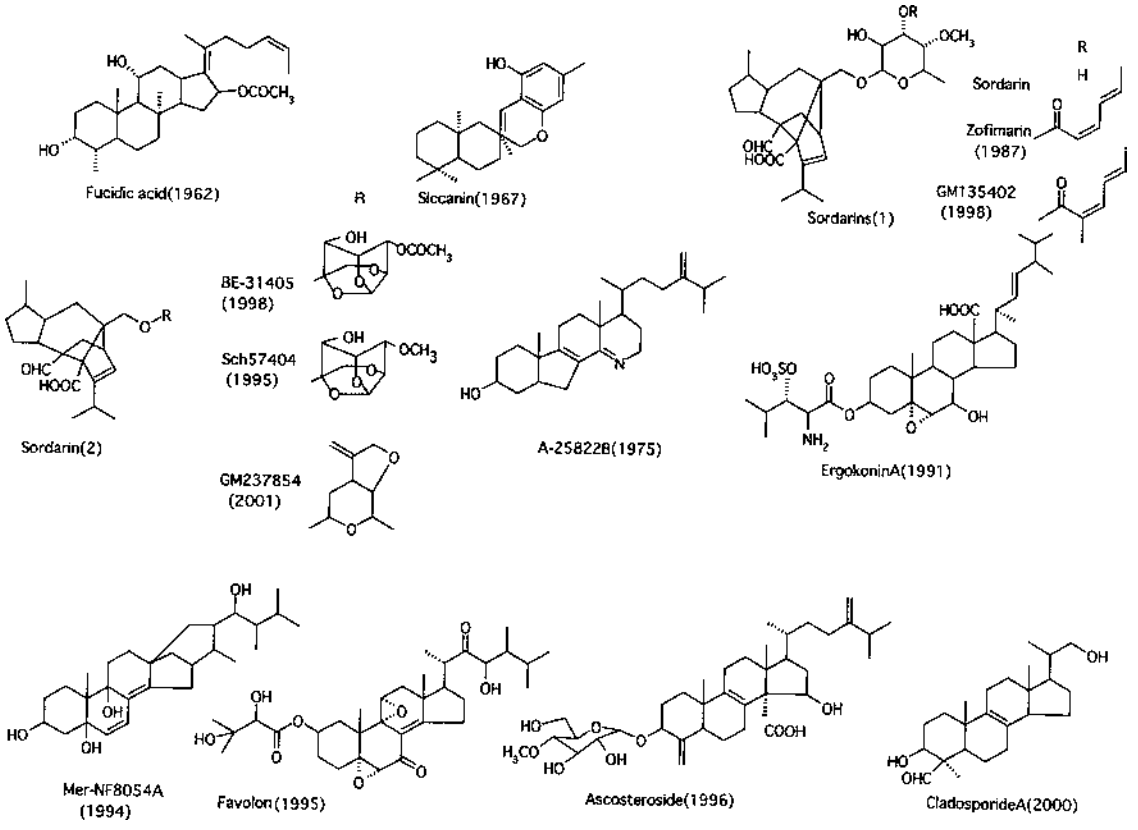

Figure 4 Chemical structures of fungal terpenoid antibitoics.

narrow, being active only against several strains of *A. fumigatus* (MIC, 0.16, 0.63 μg/ml). A triterpene, favolon was a metabolite of basidiomycetes *Favolaschia* sp. collected from wood in a forest in Ethiopia (Anke et al. 1995). Favolon exhibited potent antifungal activity against ascomycetes, basidiomycetes, oomycetes, and zygomycetes, but showed no antibacterial activity. No cytotoxicity was observed on L-1210 cells.

PF1032 A and B were discovered by the Meiji Seika research group in the culture of a soil fungus, *Neospartorya* sp. (Gomi et al. 1994). They were active against *Candida glabrata* and *C. neoformans*, but inactive against *C. albicans* and bacteria. It was found later that PF1032 reduced serum cholesterol and triglyceride by oral and parenteral administrations (Harimaya et al. 1996). Cladosporides A-D, new pentanorlanostane derivatives, were isolated from *Cladosporium* sp. (Hosoe et al. 2000). Cladosporide A exhibited a high potency against *A. fumigatus* (MIC, 0.5–4.0 μg/ml), but no activity against other filamentous fungi or pathogenic yeast, similar to Mer-NF8054A. A structure–activity study revealed that 4-β-aldehyde and 3-β-hydroxy (but not 3-α-hydroxy) groups are essential for the activity.

Merck scientists have discovered four acidic terpenoids; ergokonin A, ascosteroside, arundifungin, and enfumafungin, in the course of a screening program of glucan synthase inhibitors with improved bioavailability by oral dosing (Onishi et al. 2000). Ergokonin A, a sulfated carboxysteroid, was first isolated from *Trichoderma koningii* in 1991 (Angustiniak et al. 1991), and from *T. viride* (1994), and rediscovered by the Merck scientists from *T. longibrachiatum* (Vicente et al. 2001). Ascosteroside, closely related to PF-1032 in structure, was first discovered by the Bristol-Meyers Squibb research team (Gorman et al. 1996). The producing organism, an ascomycetous fungus *Ascotricha amphitricha*, was isolated from soil in Kenya, Africa. Ascosteroside was discovered independently from the genus *Ellisiodothis* by a joint research team of Meiji Seika and Mitsubishi Kasei, Japan under the name of MK6059 (Konno et al. 1997). Arundifungin, a new steroid, was obtained from *Arthrinium arundinis* collected in Costa Rica. Nine other fungal isolates unrelated ecologically or taxonomically produced arundifungin. Two of them were endophytes collected in central Spain (Cabello et al. 2001). Enfumafungin, another new pentacyclic terpenoid, was obtained from *Hormonema* sp., which was an endophyte isolated from leaves of *Juniperus communis* (Pelaez et al. 2000).

All these acid terpenoids were found to inhibit (1,3) β-D-glucane synthase, like echinocandin and papulacandin antibiotics, but did not inhibit steroid biosynthesis. Among

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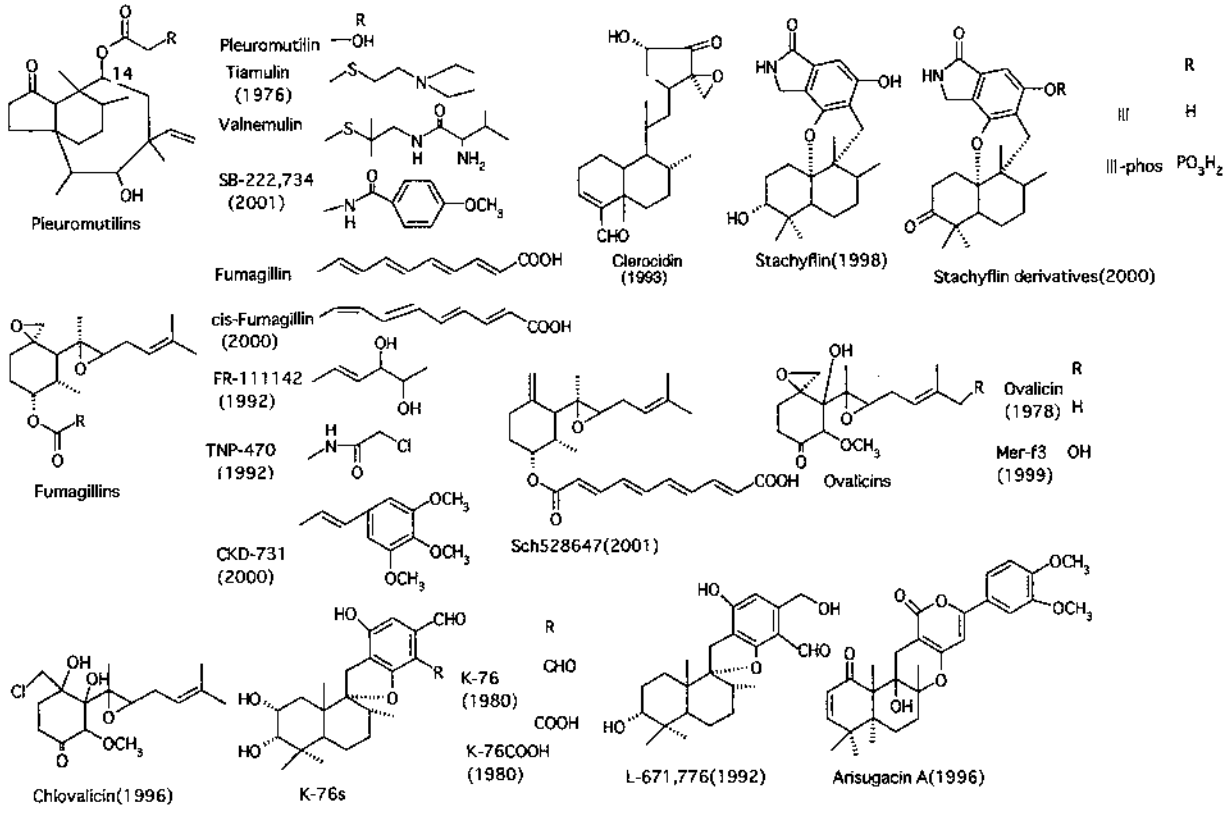

Figure 4 (*continued*).

four terpenoids, enfumafungin exhibited the most potent antifungal activity against *Candida* and *Aspergillus* spp., which was comparable to that of the semisynthetic lipopeptide, MK0991 (caspofungin acetate) (Onishi et al. 2000). Similar to MK0991, enfumafungin was inactive against *C. neoformans* and bacteria. The other three terpenoids have a similar antifungal spectrum to enfumafungin, but were less potent. Ascosteroside showed no cytotoxicity to mammalian cells, and was effective *in vivo* in a mouse candidiasis, comparable to ketokonazole (Gorman et al. 1996). Enfumafungin was also active in mouse *Candida* model by oral dosing (Onishi et al. 2000). Therefore, development of orally active (1,3) β-glucan synthase might be expected for acidic terpenoids, which appears difficult to realize for lipopeptide antibiotics.

3.2 Antibacterial Terpenoids

The emergence and spread of resistance to existing antibiotics is of major concern worldwide. There is, therefore, an urgent need to exploit new classes of antimicrobial agents with a novel mechanism of action. A tricyclic diterpene antibiotic, pleuromutilin was first isolated in 1951 by Kavanagh and coworkers from the basidiomycete, *Pleurotus mutilus*, which is now called *Clitopilus scyphoides*. The same antibiotic was isolated from *Clitopilus passeckerianus* in 1976 (Knauseder and Brandl 1976); it shows high antibacterial activity, principally against gram-positive bacteria and mycoplasma. Pleuromutilin exerts its antibacterial activity by inhibiting bacterial protein synthesis through an interaction with the rRNA in the peptidyltransferase slot on the prokaryotic ribosomes. For the development for chemotherapeutic application, a series of derivatives were prepared by the Sandoz research group during 1970–1980. As a result, tiamulin and valnemulin were developed as veterinary drugs (Egger and Reinshagen 1976). Recently, tiamulin was found to sensitize the resistant cancer cells in the presence of anticancer drugs, antagonizing the P-glycoprotein mediated chemoresistance (Baggetto et al. 1998). Econor, which contains the active ingredient valnemulin, was effective against resistant mycoplasma infection in immunocompromised patients (Heilmann et al. 2001).

The SmithKline Beecham research group has synthesized a new series of mutilin 14-carbamate derivatives, in which the 4-methoxybenzoylcarbamate, SB-222734 displayed potent antibacterial activity including resistant strains (MIC90, *Staphylococcus aureus* 0.06, *Streptococcus pneumoniae* 0.5, *Haemophillus influenzae* 2 μg/ml). Furthermore, SB-222734

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showed enhanced metabolic stability *in vivo* (Brooks et al. 2001).

The Bristol-Meyers Squibb research group discovered clerocidin, a sesquiterpenoid antibiotic by screening the bacterial DNA gyrase inhibitor (type II topoisomerase), which is the target of the quinolone antibacterial agent (McCullough et al. 1993). Clerocidin was originally isolated from *Oidiodendron truncatum* by Anderson and Rasmusin in 1984, and later from *Fusidium viride*. Clerocidin exhibited a broad and potent antibacterial activity including *Pseudomonas aeruginosa*, equal to or greater than that of ciprofloxacin. However, it has cross-resistance to quinolone antibiotics and strong cytotoxicity.

3.3 Antiviral Terpenoids

Human influenza virus types A and B cause infectious disease of the respiratory tract with severe symptoms. Amantadine and zanamivir, both synthetic drugs, are used in clinics, but there is a strong demand for development of more effective antiinfluenza agents.

Stachyflin, a pentacyclic sesquiterpenophenol, was discovered by the Shionogi scientists in 1998 (Minagawa et al. 2002a). The producing organism, *Stachybotrys* sp. was isolated from a leaf from an unidentified decaying broad-leaves tree in Amami-Oshima Island, Japan. It produced stachyflin and acetylstachyflin by solid-state fermentation, but produced by liquid fermentation bisabosuqal, a SQS inhibitor of phenylterpenoid. Alteration of condition from liquid state to solid state seemed to change the gene expression induced in the secondary production. *Stachybotrys* sp. may have at least two types of sesquiterpene cyclases, and their expression may be controlled by fermentation conditions. It is known that *Stachybotrys* organisms and a closely related fungus *Memnoniella echinata* produced a variety of metabolites of sesquiterpene polyketides with substituted aromatic rings.

Stachyflin is effective against human influenza A virus by inhibiting the fusion process between the viral envelope and the host cell membrane at an early step in the entry of virus into host cells. The antiviral activity of stachyflin against influenza A/WSN/33 virus was 1760 times more potent than amantadin and 250 times more potent than zanamivir, showing IC50 of 3 nM.

Novel derivatives of stachyflin III (an oxidation product of stachyflin) (Minagawa et al. 2002b) and III-phos (phosphate ester of III) were prepared by the Shionogi scientists. They lacked mutagenicity and showed activity against fresh human clinical isolates (Yoshimoto et al. 2000). *In vivo* anti-influenza-virus activity of III and III-phos was evaluated in mouse and ferret influenza virus infection models. Oral administration of both derivatives significantly inhibited virus replication in lungs of infected mice, and intranasal dosing of III-phos inhibited influenza virus infection in ferrets.

3.4 Antitumor Terpenoid

The concept of treating cancer by inhibition of angiogenesis (new blood vessel formation) is a promising therapy strategy. A sesquiterpenoid, fumagillin, was originally isolated from *A. fumigatus* as an antibacteriophage agent (1949) and had antiamoeba activity (1982). In 1990, Ingber et al. rediscovered fumagillin as an inhibitor of endothelial cell growth and angiogenesis (Ingber et al. 1990). Two research groups have demonstrated that the methionine aminopeptidase type 2 (MetAP-2) is a common molecular target for fumagillin, ovalicin and TNP-470, all of which are well-known angiogeneous inhibitors (Griffith et al. 1998; Sin et al. 1997). The x-ray structural analysis of the human MetAP-2 complexed with fumagillin showed that a covalent bond was formed between a ring epoxide group of fumagillin and a specific histone residue (His-231) in the active site of the enzyme (Griffith et al. 1998).

By selective screening for a new MetAP-2 inhibitor, *cis*-fumagillin was found in the culture of a soil fungus, *Penicillium janczewski* (Kwon et al. 2000). *Cis*-Fumagillin was isolated as methyl ester after treating the broth with diazomethane, as was fumagillin methylester. *Cis*-Fumagillin methylester inhibited the MetAP-2 with an IC50 value of 6.3 μM, but did not inhibit MetAP-1. Fumagillin methylester showed IC50 of 9.2 and 105 μM, respectively. The equipotency of cis- and trans-isomers, together with the x-ray analysis mentioned earlier, suggested that modification of the side chain including stereochemical configuration may be possible without a loss of bioactivity to obtain more potent antiangiogenic agents.

FR-111142 with a structural nucleus of fumagillin was discovered by the Fujisawa research group (Otsuka et al. 1992). The producing organism was a fungus *Scolecobasidium arenarium* isolated from decaying wood debris collected at a beach in Japan. It inhibited endothelial cell proliferation *in vitro*, and angiogenesis in the growing chick chorioallantoic membrane model *in vivo*.

The Schering-Plough research group has isolated a minor component of fumagillin, Sch528647 in the process of large-scale preparation of fumagillin by *A. fumigatus* for evaluation (Chu et al. 2001). The antitumor activity of Sch528647 was >31-fold less than that of fumagillin, indicating the importance of the spiroepoxide function for activity, which is changed to exomethylene group in Sch528647.

Among many semisynthetic derivatives from fumagillol, a hydrolysis product of fumagillin, TNP-470 synthesized by the Takeda research group (Marui et al. 1992) was entered in clinical trials for the treatment of a variety of cancers including Kaposi sarcoma, breast, cervical, lung, and renal cancers (Stradler et al. 1999). Based on structure-based molecular modeling with a human MetAP-2, cinnamic acid ester derivative (CKD-731) was prepared and showed 100-fold more potent proliferation inhibitory activity on endothelial cells than TNP-470 (Han et al. 2000).

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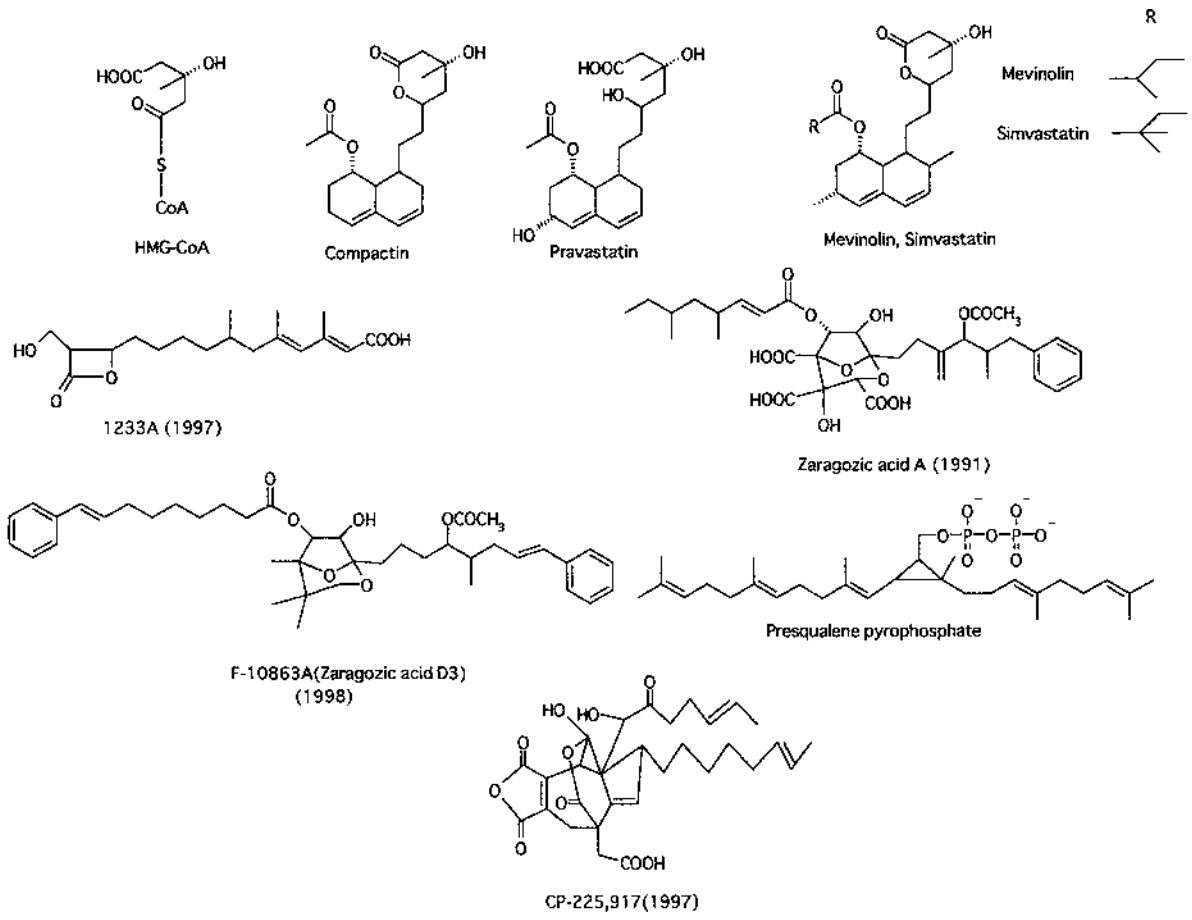

Figure 5 Chemical structures of fungal terpenoids and structurally related metabolites with enzyme inhibitory activity against human isoprenoid pathways.

Ovalicin, a sesquiterpene with structure analogous to fumagillin, was a metabolite of the ascomycete *Pseudeurotium ovalis*, which was discovered first as a lettuce seed germination stimulant (1978). It was found later to have antitumor and immunosuppressive activity (Hartman et al. 1978). It indirectly inhibited DNA synthesis in proliferating lymphocytes and in lymphoma cells at 0.1 nM, and markedly decreased the number of antibody-producing cells. The graft vs. host reaction was also distinctly delayed by ovalicin.

Another ovalicin-related sesquiterpene designated Mer-f3 (12-hydroxy-ovalicin) was isolated from the culture filtrate of a soil fungus *Metarrhyium* sp. (Kuboki et al. 1999). Mer-f3 showed a potent inhibition against human umbilical vein endothelial cells (IC50, 3.5 nM), in a degree comparable to that of fumagillin (IC50, 2.6 nM). It also showed a potent immunosuppressive activity on a mixed lymphocyte culture reaction comparable to that of ovalicin.

Chlovalicin, a chlorinated sesquiterpene derived from the epoxide ring attached to ovalicin, was discovered in the fermentation broth of a soil fungus *Sporothrix* sp. (Hayashi et al. 1996). It inhibited the IL-6 dependent cell growth of MH60 cells, and appeared to be a new type of IL-6 inhibitor. The IC50 value was 5.7 μM for MH60. The nematode-trapping fungus, *Arthrobotrys oligospora* produced oligosporon, which showed only weak cytotoxicity in spite of structural similarity to ovalicin, but did show nematocidal activity (Anderson et al. 1995). Thus, a variety of biological activities were demonstrated for ovalicinlike metabolites.

3.5 Other Bioactive Terpenoids

The complement system participates in many unfavorable reactions by producing allergenic effects, though it contributes greatly to the host defense by killing pathogens and promoting phagocytosis. Thus, it is expected that blocking of complement activation may prevent many allergic reactions and immune-complex diseases.

K-76 is a benzodrimane skeleton-containing sesquiterpenoid in which a benzene ring is attached to terpenoid through a spirofuran. K-76 was discovered by the Otsuka

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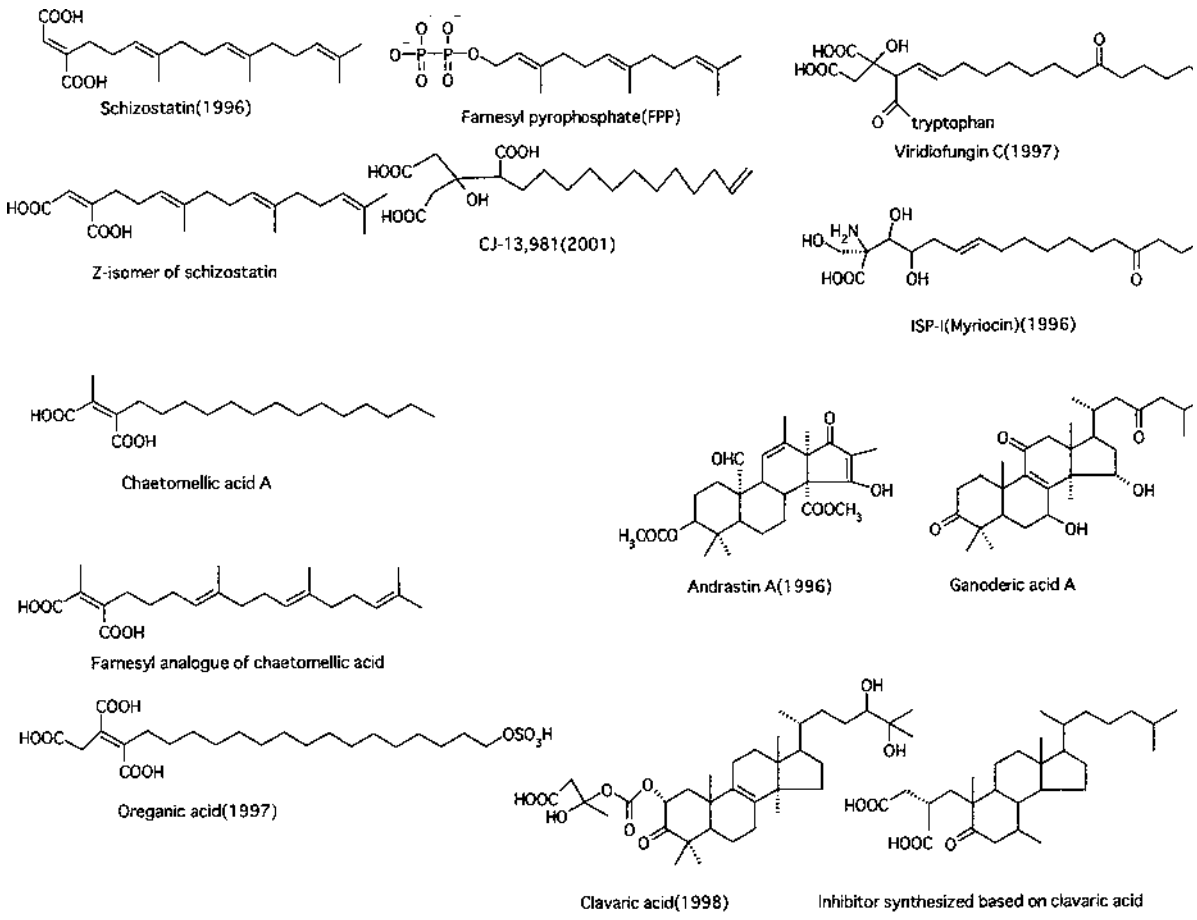

Figure 5 (*continued*).

Seiyaku research group from *Stachybotrys complementi*, which was isolated from soil on Ishigaki Island, Japan (Miyazaki et al. 1980). K-76 blocks both classical and alternative pathways of complement activation.

K-76 COOH was prepared from K-76 by partial oxidation of one of two aromatic aldehyde groups. It is readily soluble in water as its sodium salt is less toxic and does not inhibit biologically important proteases or esterases. It blocked complement activation specifically at the C-5 intermediate step of the complement activation cascade. It further suppressed Forssman shock in guinea pigs and mice, and heterologous passive cutaneous anaphylaxis in guinea pigs (Miyazaki et al. 1984). It was clinically evaluated as a new drug for treatment of active stage ulcerative colitis (Kitano et al. 1992). L671,776 (Factor B) produced by *M. echinata* has a structure very similar to K-76. It showed inositol monophosphatase inhibition (Lam et al. 1992), similar to K-76 COOH (Pachter 1991).

A current clinical study with the acetylcholine esterase (AchE) inhibitor tacrine has demonstrated a significant improvement of cognitive function in patients of Alzheimer's disease (AD). Tacrine has been approved by the FDA as the first agent for the treatment of AD, although it does have dose-limiting side effects. The peripheral side effect was assumed associated with butyrylcholine esterase (BchE) inhibition. Therefore, selective AchE inhibition over BchE would be promising for treating AD.

Arisugacin, a sesquiterpenoid, was discovered in the culture broth of a soil fungus, *Penicillium* sp. in the screening of AChE inhibitor by Kitasato scientists (Kuno et al. 1996). Arisugacin specifically inhibited AchE (from human erythrocytes) with IC50 values of 1.0 nM, but did not inhibit BchE (from horse serum). The inhibitory activity of arisugacin against AchE was 200 times stronger than that of tacrine, while the inhibitory activity against BchE was more than 1,500 times weaker than that of tacrine. Highly selective and potent inhibitory activity of arisugacin against AchE suggested possible application for treating AD. Interestingly, arisugacin resembles pyripyropene, which is a potent ACAT inhibitor, but the correlation of structures and AchE and ACAT inhibitions is not clear.

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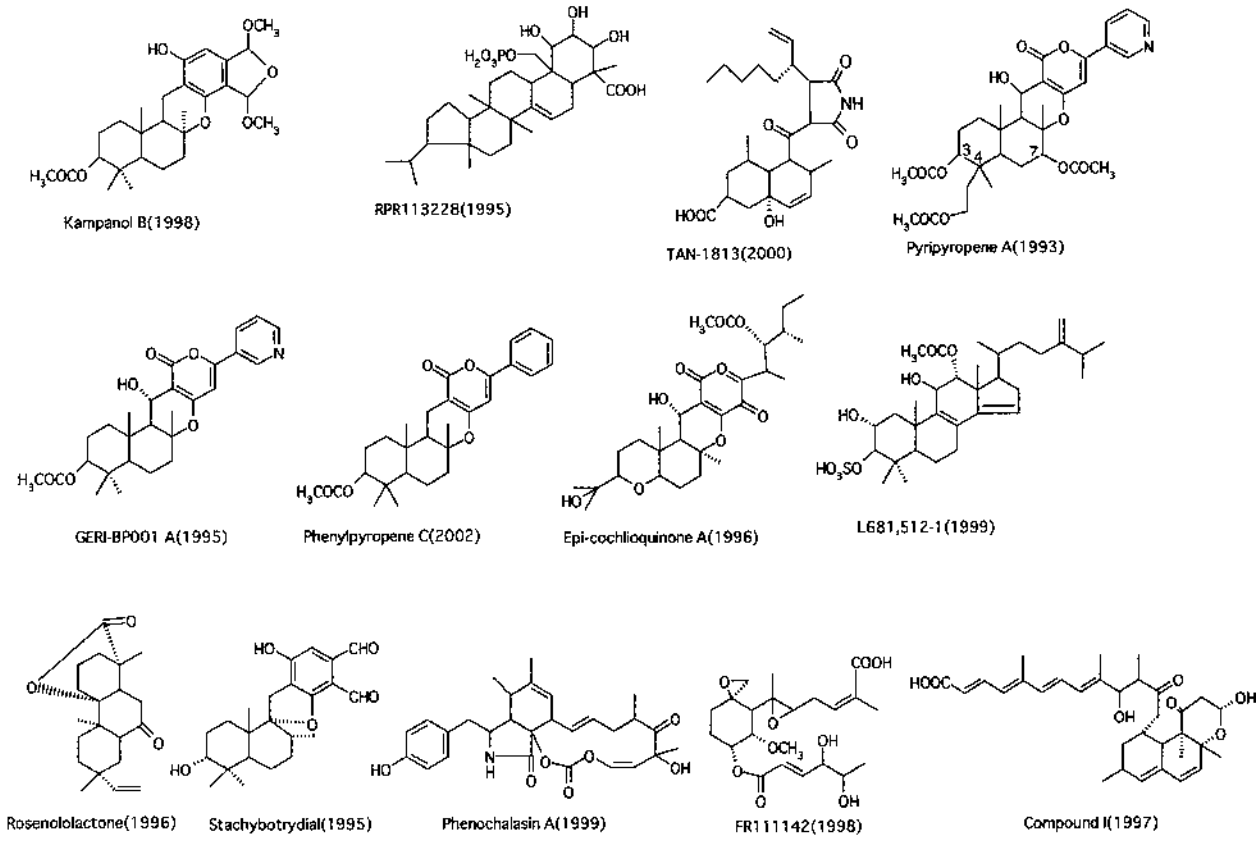

Figure 5 (*continued*).

4 FUNGAL TERPENOIDS AND STRUCTURALLY RELATED METABOLITES WITH THE ENZYME INHIBITORY ACTIVITY AGAINST HUMAN ISOPRENOID PATHWAYS

The chemical structures of enzyme inhibitors described later are shown in Figure 5.

4.1 3-Hydroxy-3-Methylglutaryl-Coenzyme A (HMG-CoA) Reductase and Synthase Inhibitors

HMG-CoA reductase reduces the HMG-CoA to the six-carbon mevalonate, and catalyzes the rate-limiting step in cholesterogenesis and hypercholesterolemia, which are known primary risk factors for coronary heart diseases. Therefore, HMG-CoA reductase inhibitors have been a target of extensive search for pharmaceutical laboratories. Research and development of pravastatin by the Sankyo research group and simvastatin by the Merck research group are well known, and only briefly described here. Compactin (ML-236B) obtained from *Penicillium citrium* and *P. brevi-compactum* was converted to pravastatin by microbial hydroxylation (Kishida et al. 1991). Lovastatin (mevinolin, monakolin K) obtained from *Aspergillus tereus* and *Monascus ruber* was converted to simvastatin by chemical modification (Hoffman et al. 1986). Pravastatin and simvastatin, as well as compactin and mevinolin, are competitive inhibitors of HMG-CoA reductase, and reduced total cholesterol and decreased LDL level by increasing the number of LDL receptors in animals and human (McTavish and Sorkin 1991).

Compactin and mevinolin are composed of two polyketid chains and mimics of HMG-CoA reductase: a simple diketide as a mimic of HMG, and hexahydro-naphthalene ring as a mimic of CoA (Figure 5). Recently, an analog of compactin (3-α-hydroxy-3,5-dihydro-ML-236C) was isolated from *Paecilomyces viridis* (Murakawa et al. 1994).

HMG-CoA synthase committed in the first step of the isoprenoid biosynthesis is an important site of control for the cholesterol pathway. Following the discovery of HMG-CoA reductases, a worldwide effort was made to discover agents that have a specific inhibitory activity on HMG-CoA synthase.

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1233A was first isolated as an antibiotic from *Cephalosporium* sp. by Aldridge et al. in 1971, and rediscovered as a HMG-CoA synthase inhibitor by Kitasato scientists from *Scopulariopsis* sp. and by Merck scientists from *Fusarium* sp. independently in 1987 (Greenspan et al. 1987; Tomoda et al. 1988). 1233A specifically and irreversibly inhibited HMG-CoA synthase with an IC50 value of 0.12 nM. It inhibited overall synthesis of steroids, but did not inhibit HMG-CoA reductase, β-ketoacyl-CoA thiolase, acetoacetyl-CoA synthase, or fatty acid synthase.

Study of the structure-activity relationships revealed that the β-lactone ring must be intact for activity, and that the trans geometry of β-lactone is important, since the *cis*-β-lactone is inactive. The enzyme inhibition was due to the β-lactone ring opening by the active site of cystein to form an enzyme bound thioester (Mayer et al. 1990).

4.2 Squalene Synthase (SQS) Inhibitors

Squalene synthase occupies a key branch point in the isoprenoid pathway, catalyzing the first committed step of steroids. Selective SQS inhibitors, therefore, are expected to reduce steroids without affecting essential nonsteroidal products. In 1991, the Merck research group discovered zaragozic acid in the culture of an unidentified fungus isolated from a water sample in Zaragoza, Spain (Bergstrom et al. 1995). At the same time, the Glaxo research group independently discovered squastatins from the culture of *Phoma* sp. (Dawson et al. 1992). It was later found that squastatin 1 was identical to zaragozic acid A, squastatin II to 4′-desacetyl zaragozic acid A, and so on. Zaragozic acid A (squastatin I) has also been isolated from *Setospharia khartoumensis* (Hasumi et al. 1993). A large number of minor members of zaragozic acid/squastatins were isolated, and bioconverted products were prepared by the Merck and Glaxo groups. Now, it is apparent that the zaragozic acids are a large family of fungal metabolites produced by various filamentous fungi, most of which are either ascomycetes or their anamorphs or sterile fungi with ascomycete affinities. The zaragozic acid-producing fungi have been reported to occur in water, bark, stems, dead wood, a corticolous lichen, a basidioma, dung, and soil.

The bicyclic backbone of zaragozic acids is not a terpenoid, but it is built up by the polyketide pathway. The structure consists of a highly acidic central core connected with two long hydrophobic tails and mimics presqualene pyrophosphate (PSPP), a product of the first step in squalene synthesis (Figure 5). Zaragozic acid appeared to inhibit squalene synthesis by effectively mimicking the binding of PSPP to the SQS. Both the first and second steps in the squalene synthesis appeared to be inhibited by this acid. The SQS inhibitory activity of zaragozic acid A was very potent, with an IC50 value of 78 pM. The inhibition was competitive with respect to the substrate FPP. When administered intravenously or subcutaneously in mice or rats, or when dosed orally in marmosets, zaragozic acid inhibited cholesterol synthesis *in vivo*.

All zaragozic acids have potent antifungal activity as well. The activity appears to result from the inhibition of squalene synthesis, but the strong binding of these acids to albumin and their rapid delivery to liver may limit their utility.

In 1997, the Sankyo research group isolated from the culture broth of the fungus *Mollisia* sp. novel zaragozic acids, F-10863 A, B, C, and D (Tanimoto et al. 1997). F-10863 A was identical to zaragozic acid D3, which was produced by *Libertella* sp. F-10863 A and B are strong SQS inhibitors *in vitro*, though their inhibition of sterol synthesis was weak in the cells tested except for hepatic cells. Similar to zaragozic acid, F-10863 reduced serum cholesterol levels in hamsters and marmosets when administered orally. However, the inhibition of SQS caused acidosis due to the accumulation of farnesol-derived dicarboxylic acids in urine.

CP-225,917 and CP263,114 were isolated from an unidentified fungus collected from twigs in a juniper-scrub oak forest, in Texas by the Pfizer research group (Dabrah et al. 1997). The producing organism was close to a sterile *Phoma* species. It has some structural similarity to zaragozic acid that was produced simultaneously. CP-225,917 inhibited the enzymatic first reaction, and showed IC50 of 43 μM against SQS, considerably weaker than zaragozic acid A. Inhibition was of the mixed noncompetitive type with respect to the substrate FPP, suggesting that this compound can bind with different affinities to both the free enzyme and the enzyme-FPP complex. CP-225,917 inhibited Ras PFT more potently than it inhibited SQS, with IC50 of 6 μM. Zaragozic acid D and D2 inhibited PFT with IC50 of 100 nM (Dufresne et al. 1993). In general, zaragozic acid was found to be competitively inhibitory against Ras PFT with respect to FPP, but the inhibitory potency was 100–2000 fold less than that of SQS.

Schizostatin with a structure completely different from zaragozic acids was discovered by the Sankyo research group (Tanimoto et al. 1996). The producing fungus was a mushroom, *Schizophyllum commune*, which was isolated from a contaminated wood door. Schizostatin has a farnesyl group and a two carboxylic acid moiety in the molecule, and is a FPP mimic. It inhibited SQS with an IC50 value of 0.84 μM, and the inhibition was competitive with respect to FPP. When administered orally, schizostatin inhibited murine hepatic steroid synthesis *in vivo*.

The stereochemistry of the dicarboxylic acid moieties is closely related to the enzyme inhibition. The E-isomer (schizostatin) has more selective SQS inhibition than the Z-isomer (synthetic compound). As will be shown later, chaetomellic acid A, which has a Z-isomer configuration (cisoid head), showed potent inhibitory activity against PFT but weak or no inhibitory activity against SQS. It is interesting that the E isomer configuration is suitable for the inhibition of SQS, while the Z-isomer configuration is fitted for the inhibition of PFT.

CJ-13,981 and CJ-13,982 were isolated from an unidentified fungus by the Pfizer research group (Watanabe

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et al. 2001). CJ-13,982 is similar in structure to schizostatin, and inhibited rat liver SQS with an IC50 value of 2.2 μM, human liver SQS with 1.1 μM, but human brain PFT with >100 μM. However, it did not inhibit cholesterol biosynthesis in either Hep-G2 cells or chow-fed mice.

A family of aminoacyl alkyl citrate compounds called viridiofungins were isolated from *T. viride* by the Merck scientists in 1997 (Onishi et al. 1997). Viridiofungin possessing polyanionic functions with a hydrophobic chain are similar in structure to schizostatin and CJ-13,982. Viridiofungin C inhibited *S. cerevisiae* SQS with an IC50 value of 0.35 μM, and showed broad antifungal activity without antibacterial activity. However, more detailed study revealed that the antifungal activity was unrelated to inhibition of ergosterol synthesis, but due to inhibition of the first enzyme in the sphingolipid synthesis, the serine palmitoyltransferase (Mandala et al. 1997). Other sphingolipid synthesis inhibitors with analogous structure to viridiofungin were reported (Mandala and Harris 2000). These fungal metabolites exhibited marked antifungal activity.

4.3 Protein Prenyltransferase Inhibitors

The currently understood function for Ras in signal transduction is to mediate the transmission of signals from external growth factors to the cell nucleus. Mutated forms of this GTP-binding protein are found in 30% of human cancers with particularly high prevalence in colon and pancreatic carcinomas. The mutations destroy the GTPase activity of Ras and cause the protein to be locked in its active, GTP bound form. As a result, the signaling pathways are activated, leading to uncontrolled tumor growth. Ras function in signaling requires its association with the plasma membrane. This is achieved by posttranslational farnesylation, in which PFT catalyzes the transfer of a 15-carbon farnesyl group from FPP to the cysteine, the fourth residue from the carboxyl terminal CAAX motif of peptide and protein substrates. The fact that farnesylation of the oncogenic Ras is essential for its cell transforming activity means Ras PFT is a promising target for development of an antitumor agent. During the last decade, many types of inhibitors were discovered by microbial random screening.

There are three types of inhibitors reported to date, and these fall into three main classes: (a) inhibitors that are competitive with FPP, a substrate of PFT; (b) inhibitors that are competitive with Ras peptide; and (c) inhibitors that are either not competitive with PFT as a substrate or are of unknown mechanism.

Merck scientists have isolated two novel acidic types of PFT inhibitors, chaetomellic acid and oreganic acid, which are FPP mimicking inhibitors belonging to class (a) as shown in Figure 5. Chaetomellic acids were isolated from the coelomycete *Chaetomella acutiseta*, and inhibited Ras PFT with an IC50 value of 55 μM (Singh et al. 1993). The inhibition was highly competitive with respect to FPP, and

Table 1 Inhibitory activity of schizostatin, chaetomellic acid and related synthetic analogs against SQS, PFT and PGGT

	IC50 (μM) [Tanimoto et al. 1996]	
Inhibitor	SQS	PFT
Schizostatin	0.84	no Inhibition
Z-Isomer of schizostatin	12	Weak
Chaetomellic acid	Weak	0.055

	IC50 (μM) [Ratemi et al. 1996]	
Inhibitor	PFT	PGGT
Chaetomellic acid (CA)	17	>300
Farnesyl analog of CA	2.4	277
Geranylgeranyl analog of CA	96	11.5

noncompetitive with respect to acceptor peptide Ras, suggesting that it was mimicking the FPP at the active site. Chaetomellic acid and FPP have structural similarity as they both possess a hydrophilic head group and hydrophobic tail group (Figure 5). When the tetradecyl chain of chaetomeric acid was replaced chemically with a farnesyl moiety, potency of the inhibitory activity of PFT was increased 7-fold against yeast PFT, and more selective to PFT than geranylgeranyltransferase (PGGT) of yeast as shown in Table 1 (Ratemi et al. 1996). When replaced with a geranylgeranyl side chain, the inhibitory activity was changed in favor of PGGT.

Chaetomellic acid A was a specific human PFT inhibitor that did not inhibit human SQS. Structurally-related CJ-13,982 showed a high specificity for SQS inhibition relative to PFT. The structural difference between the two inhibitors is that chaetomellic acid A has methylenmaleic acid moiety whereas CJ-13,982 has a citric acid moiety. This suggested that while SQS and PFT both use FPP as a substrate, the substrate-binding pocket on each enzyme can effectively discriminate between the polyanionic functions.

Oreganic acid was isolated from an endophytic fungus from the leaves of *Berberis oregana* (Silverman et al. 1997). It exhibited potent and reversible inhibition against recombinant human PFT with an IC50 value of 14 nM, and showed an exquisite selectivity for FPT over PGGT-1 (IC50, 60 μM). Oreganic acid is more potent than chaetomellic acid in the inhibitory activity. Trimethylation of the carboxylic groups or desulfation led to a significant decrease in PFT inhibitory activity, indicating that ionizing groups of both carboxylic acid and sulfuric acid are essential for the activity. Unfortunately, oreganic acid did not affect Ras processing in whole cell assays. This is probably due to the charged nature of the compound that precludes its entry into the cell.

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Another series of fungal metabolites possessing a hydrophilic head and a hydrophobic tail were isolated. Those included ISP-I (myriocin, thermozymocin) and mycesterins. ISP-1 was discovered from *Isaria sinclairii* (Fujita et al. 1994). Though its structure is similar to the other SQS and PFT inhibitors chaetomellic acid and schizostatin, it is a potent immunosuppressant. It was 10–100 times more potent than cyclosporin A in terms of suppressing lymphocyte proliferation in mouse allogeneic mixed lymphocyte reaction *in vitro*, and allo-dependent antibody production *in vivo*. Mycestricin A produced by *Mycelia strilia* was also an immunosuppressant (Sasaki et al. 1994).

Several steroidlike PFT inhibitors were isolated from fungi. Andrastins were isolated from a soil fungus *Penicillium* sp. by Kitasato and Keio University scientists (Omura et al. 1996). Andrastins were not originated solely from isoprenoid units, but were biosynthesized from a sesquiterpene and a tetraketide called meroterpenoid. Andrastin C reversibly inhibited human PFT with an IC50 value of 13.3 μM. Recently, andrastin A was rediscovered as a compound that reverses multidrug resistance (Rho et al. 1998). The cytotoxicity of vincristine was enhanced 1.5–20 fold by andrastin A in vincristine-resistant KB cells, by directly interacting with P-glycoprotein to inhibit the efflux of vincristine. It is assumed that PFT and P-glyco protein function are not related.

The fruiting body of *Ganoderma lucidum* (Reishi in Japanese) is a well-known Chinese drug, and over one hundred highly oxygenated lanostane-type triterpenes have been isolated from the fruiting bodies and mycelia of *G. lucidum*. Among them, ganoderic acid A showed moderate inhibitory activity against PFT with an IC50 value of 100 μM (Lee et al. 1998).

The Merck research group discovered two steroidal analogs of PFT inhibitors. Clavaric acid was isolated from a basidiomycetes *Clavariadelphurs fruncatus* (Jayasuriya et al. 1998). Kampanols were isolated from a fungal culture *Stachybotrys kampalensis* collected from leaf litter in Costa Rica (Singh et al. 1998). Clavaric acid and kampanol B specifically inhibited human PFT with IC50 values of 1.3 and 7 μM, respectively. The inhibition was reversible for FPP, and competitive with respect to Ras, but no inhibition was observed against PGGT. No SQS inhibition was observed with clavaric acid. As a result of computer-based similarity searches and subsequent rational chemical synthetic design, Merck scientists provided a highly potent inhibitor (Figure 5) (IC50, 40 nM) (Lingham et al. 1998).

RPR113228 was isolated from a soil fungus *Chrysosporium lobatum* by Rhone-Poulenc Rore scientists (Van der Pyl et al. 1995). Inhibition of human PFT was IC50 of 0.83 or 2.1 μM, competitive with respect to FPP while having weak inhibition against human PGGT (IC50, 59 μM) and no inhibition against rat liver SQS.

The Takeda research group discovered TAN-1813 with a unique imido group in the culture broth of a plant endophyte fungus, *Phoma* sp., which was isolated from a root of *Erigeron annus* in Japan (Ishii et al. 2000). TAN-1813 inhibited Ras PFT with IC50 of 23 μg/ml, and PGGFT with IC50 of 47 μg/ml. The inhibition was competitive with FPP, but noncompetitive with GGPP. TAN-1813 showed mixed type inhibition with respect to FPP and was noncompetitive with respect to a K-Ras C-terminal peptide. A C8-alkenyl moiety of TAN-1813 was assumed responsible for the competition. TAN-1813 was less effective against PFT of other small G proteins (Rho, Rab) to which Ras belonged. TAN-1813 inhibited HT-1080 and NIH3T3/k-ras tumors harboring oncogenic ras gene, but not P-388 or Lewis lung carcinoma harboring no oncogenic ras alleles. Many microbial PFT inhibitors have not been shown to inhibit Ras-dependent tumor growth *in vivo*, but TAN-1813 reduced Ras-dependent tumor growth in a nude mouse model.

Although no fungal terpenoid-inhibiting PFT has been developed so far as an antitumor agent, synthetic PFT inhibitors Sch66336 and R115777 have entered clinical trials. Moreover, two monoterpenes, limonene and peryllalcohol, which inhibited PFT with IC50 of 5 and 1 mM, respectively, (Crowell et al. 1994), are currently being evaluated in clinical study for therapeutic efficacy against pancreatic and breast cancers (Crowell et al. 1996). Although these monoterpenes are of plant origin, many fungi are also capable of producing limonene.

Protein prenylation also occurs in trypanosomatids and probably apicomplexan parasites. Although no fungal inhibitor was discovered, synthetic inhibitors of FPP synthase, such as bisphosphonates, were shown to be active *in vitro* and *in vivo* against parasitic protozoa (Martin et al. 2001; Yardley et al. 2002). It was assumed that inhibition of FPP synthase caused the decrease in protein prenylation as well as the decrease of dolichol, ubiquinone, and sterol formation, leading to parasite growth inhibition.

4.4 Acyl-CoA:Cholesterol Acyltransferase (ACAT) Inhibitors

Acyl CoA: cholesterol acyltransferase is responsible for intracellular esterification of cholesterol, and plays a key role in intestinal absorption of cholesterol, hepatic production of lipoproteins, and accumulation of cholesteryl esters as oil droplets within macrophages and smooth muscle cells of the atheroma. Therefore, ACAT is an attractive target for new treatments of hypercholesterolemia and atherosclerosis.

A large number of pyripyropenes consisting of sesquiterpene, α-pyrone and pyridine moieties, were discovered by Kitasato scientists from the culture of *A. fumigatus* (Tomoda et al. 1994). The sesquiterpene moiety was suggested to be produced via cyclization of an epoxidated farnesyl intermediate. So far, 18 homologues of pyripyropene A have been isolated, and more than 300 derivatives synthesized. Pyripyropene A, the principal metabolite, showed the most potent inhibitory activity against ACAT of rat liver microsomes with an IC50 value of 58 nM. A study of structure-activity relationships revealed that 7-*n*-valeryl-7-deacetyl pyripyropene A showed 7-fold more potent ACAT

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inhibition than the parent A, with IC50 of 13 nM (Obata et al. 1995). On the other hand, 7-benzoyl-pyripyropene A was claimed to reverse cancer cell multidrug resistance and to show cytotoxicity to P-388 (Rho et al. 2000). Pyripyropene A was also obtained from *Eupenicillium reticulisporum* in antiinsectan screening (Wang et al. 1995).

Three other sesquiterpene ACAT inhibitors with analogous structures to pyripyropenes were isolated. GERI-BP001A was discovered from a soil fungus *A. fumigatus* in South Korea (Jeong et al. 1995), and inhibited ACAT of rat liver microsomes with IC50 of 94 μM, a 1000-fold less than that of pyripyropene A, in spite of their structural similarities. Differing from pyripyropene A, GER-BP001 lacked acyloxy groups at C-4 and C-7, which apparently significantly contributed to the inhibitory activity.

Phenylpyropene C was discovered in the culture broth of a soil fungus *Penicillium griseofulvum* (Rho et al. 2002). It inhibited ACAT with an IC50 value of 16.0 μM. The IC50 value of pyripyropene E was reported to be 399 μM. Since the pyridine ring of pyripyropene E was replaced by the benzene ring in phenylpyropene C, a benzene ring appeared to be an effective substitute as a pyridine ring for the inhibitory activity.

Epi-cochlioquinone A was isolated by Sankyo scientists from the fermentation broth of *Stachybotrys bisbyi* (Fujioka et al. 1996). It inhibited ACAT activity in an enzyme assay using rat liver microsomes with an IC50 value of 1.7 μM.

So far, only synthetic ACAT inhibitors like F-1394 have been tested in a clinical study.

4.5 Cholesterylester Transfer Protein (CETP) Inhibitors

Atherosclerogenesis is caused by the deposition of lipids, especially cholesterol, onto arterial cells, and continued lipid deposition leads to coronary heart disease. Epidemiological studies showed that the risk of coronary heart disease correlates with increased cholesterol levels associated with LDL, and inversely correlates with increased cholesterol levels associated with HDL. Cholesterylester transfer protein is a plasma protein that mediates the exchange of cholesteryl ester in HDL for triglyceride in VLDL. This process decreases the level of antiatherogenetic HDL cholesterol and increases pro-atherogenic VLDL and LDL cholesterol, so CETP is potentially atherogenic. On the other hand, CETP could also be antiatherogenic because it participates in reverse cholesterol transport (transfer of cholesterol from peripheral cells through the plasma to the liver). Evidence is accumulating for the involvement of CETP in atherosclerosis (Qian et al. 1997). Inhibition of CETP by a synthetic JTT-705 led to elevation of HDL cholesterol and reduction of non-HDL cholesterol in rabbits (Okamoto et al. 2000). JTT-705 is being evaluated in clinics as an antiatherosclerotic agent.

L-681,512, which was originally isolated in 1989 as an elastase inhibitor showing antiinflammatory action, was rediscovered in the fermentation broth of a fungus *Fusarium* sp. as CETP inhibitor by Kitasato and Pfizer scientists (Tabata et al. 1999). L-681,512s are a mixture of triterpenes with sulfate and acyl groups. Among them, L-681,512-1 inhibited CETP reversibly with IC50 of 13.8 μM, and showed *ex vivo* efficacy when transgenic mice expressing human CETP and human apo A-1 were used.

Rosenololactone analogs that are tricyclic diterpenes were isolated from a soil fungus *Trichothecium roseum* (Kim et al. 1996). Rosenololactone, the principal metabolite, mildly inhibited CETP with IC50 of 65 μg/ml.

4.6 Other Modulators of Cholesterol Metabolism

Cholesterol esterase in pancreatic juice binds to the intestinal mucosa, and plays a key role in the intestinal absorption of dietary cholesterol. Removal of CEase from the pancreatic juice resulted in a marked reduction in cholesterol absorption in rats, suggesting the possible suppression of serum cholesterol level (Gallo et al. 1984).

By screening for CEase inhibitors of microbial origin, the scientists of Tokyo Tanabe and Tokyo Noko University found a fungal strain, *Stachybotrys* sp. that produced a series of active metabolites including stachybotrydial and K-76 (Sakai et al. 1995). Stachybotrydial is a triprenyl phenol, and was originally isolated as a metabolite of *Stachybotrys cylindrospora* by Ayer et al. in 1993. It should be noted that stachybotrydial is a deoxy derivative of K-76, which was discovered to be a complement inhibitor. Stachybotrydial inhibited porcine pancreatic CEase with IC_{50} of 60 μM. The inhibition was time-dependent and irreversible, but it did not affect HMG-CoA reductase or ACAT activity at concentrations up to 1 mM. Oral administration of stachybotrydial in normal-fed rats and cholesterol-fed rats reduced the serum level of cholesterol. Since stachybotrydial did not inhibit ACAT, the inhibition of intestinal cholesterol absorption was ascribed to the inhibition of CEase. K-76, a complement inhibitor as described earlier, was found to be a modulator of cholesterol metabolism in the screening.

In the early stage of atherosclerogenis, macrophages penetrate the intima, efficiently take up modified LDL, store cholesterol and fatty acid in the form of cholesteryl ester and triacylglycerol in cytosolic lipid droplets, and are converted into foam cells, leading to the development of atherosclerosis in the arterial wall. Therefore, inhibitors of macrophage-derived foam cell formation are expected to retard the progression of atherosclerosis.

Using the mouse peritoneal macrophages as a foam cell formation model, Kitasato scientists have discovered phenochalasins belonging to the cytochalasan family in the culture of *Phomopsis* sp. (Tomoda et al. 1999). Phenochalasin A caused a dose-dependent reduction in the size and number of lipid droplets in mouse macrophages with no cytotoxic effect up to 20 μM. It inhibited cholesteryl ester synthesis specifically with an IC50 value of 0.61 μM (Namatame et al. 2000).

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The cholesterol that accumulates in atherosclerotic lesions originates primarily in plasma lipoproteins including LDL. It is expected that agents that enhance cellular LDL catabolism lower plasma cholesterol level, suggesting their possible utility as an anti-atherosclerosis drug. The search from microbial culture extracts by scientists of Tokyo Noko University led to the identification of FR111142 as an active metabolite (Harada et al. 1998), which was first reported as an angiogenesis inhibitor in 1992 (Otsuka et al. 1992). FR111142 is a sesquiterpene ester with analogous structure to that of fumagilin. The treatment of human hepatoma cell line HepG2 with 40 μM of FR111142 caused a 1.9 to 2.2 fold elevation of binding, internalization and degradation of LDL in the cells. FR111142 enhanced LDL catabolism by a mechanism that involves a receptor other than LDL receptor.

A new type of cholesterol-lowering agent was discovered by Merck scientists (Bartizal et al. 1997). Compound I produced by *Aspergillus versicolor* induced LDL receptor gene, which increased the number of LDL receptors resulting in reduced blood cholesterol levels, as seen by the HMG-CoA reductase. It also exhibited broad-spectrum antifungal activity. The induction of the LDL receptor gene appeared to be a highly specific and more effective treatment, compared to known agents acting solely at the cellular level. Although synthetic agents such as SCH58235 that inhibited the uptake of cholesterol through brush border of small intestine have entered clinical trials, no fungal terpenoid has been tested in such tests to date.

5 CONCLUSIONS

To identify microbial metabolites with structural diversity, it is generally accepted that a diverse and novel repertoire of microbes is desirable. Compared with actinomycetes, in which soil is the main source, fungi have the advantage of being collected from broad ecological sources. Especially, the fungi harbored in plants including endophytes, and in fewer cases, insects, have currently become a major target of collection screens, in addition to those from soil, as shown in this review. The rare fungi collected from nature are sometimes difficult to identify, since they are unlikely to produce spores in artificial media.

In vitro screening assays based on molecular mechanisms have recently become more useful as seen in this review, especially to find novel microbial metabolites with specific targets of action. This was demonstrated by the successful discovery of the antifungal family of sordarins, inhibiting yeast specific protein synthesis, and acidic steroids inhibiting fungus-specific cell wall synthesis. In contrast, many metabolites show only cell-free activity. There is a debate as to whether screening efforts should focus on highly specific mechanism-based targets using purified subcellular ligands and reagents, or be based on functional activity at the cellular, tissue or whole animal levels. Metabolites screened by cell-free assays do not always show the same activity in whole cell assays as in the case of chaetomellic acid and oreganic acid described in this review, which cannot penetrate mammalian cells. It is our opinion that whole cell assays and animal assays should be emphasized in the early stages of the screening program for the development of useful drugs.

The work reviewed here, most of which was done in the last decade, resulted in the discovery of a large number of enzyme inhibitors from fungal terpenoids and related metabolites with diverse structures. These novel inhibitors are useful as biochemical tools to study the mechanism of action of various terpenoids *in vivo*, especially cholesterol for human health. Although inhibitors of several different enzymes involved in the biosynthesis of cholesterol have been found, other enzymes remain to be targeted by the fungal screening program. Inhibitors of lanosterol 14-α-demethylase, a target of antifungal azoles such as restricticins, were isolated from fungi, but inhibitors of squalene 2,3-epoxidase, a target of antifungal allylamines, were not. PFT inhibitors were extensively searched, but PGGT inhibitors were scarcely studied. Geranylgeranyl-pyrophosphate and PGGT play an important role in the cell-cycle progression including cellular proliferation, differentiation, and apoptosis. On the other hand, the FPP synthase inhibitors were shown to be active against parasitic protozoa including *Plasmodium falciparum*. These results suggest that fungal inhibitors of FPP and GGPP formation and/or PGGT may become valuable tools in molecular biology studies, exploring new areas of therapeutic strategy. Also interesting are enzyme inhibitors of the nonmevalonate pathway which are expected to show antibiotic activity against gram-negative bacteria and malaria with high degree of selectivity because this pathway does not exist in mammals.

Although many fungal terpenoids and structurally related metabolites have been isolated, only a few of them have entered clinical trials, TNP-470 (a derivative of fumagillin) and K-76. There are two major obstacles to overcome. First, fungal terpenoids showing potent antibiotic activity frequently exhibit strong cytotoxicity as in the cases of trichothecenes and clerocidin described in this review. Although the cytotoxicity contributes to development of some antitumor agents such as fumagillins, it is a fatal disadvantage for development of other medical agents. A slight change of the structure could cause an unexpectedly high cytotoxicity. For example, phenochalasin A showed little cytotoxicity, but phanochalacin B, in which a double bond of the former compound is replaced by an epoxide, exhibited severe cytotoxicity. A25822B, originally discovered as a potent antifungal agent, was later found as a cytotoxic agent as reviewed here. Second, terpenoid compounds are abundant in nature, and may have diverse biological activities due to specific interactions with certain cellular proteins and/or cell membranes in mammals. Indeed, quite a few terpenoids showed multiple biological activities. For example, ganoderic acid showed not only an anti-PFT activity but also antinociceptive, antioxidative, and hepatoprotective activities. Furthermore, analogs of ganoderic acid A showed inhibitory activities against HIV-1

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protease, phospholipase A2, and cholesterol biosynthesis as well as cytotoxicity. Therefore, the absence of any side effects must be confirmed when the metabolite has been selected using a mechanism-based assay.

In spite of these constraints, fungal terpenoids or their derivatives such as sordarin, enfumafungin, pleuromutilin, fumagillin, and stachyflin appeared to be promising, and it is hoped they will enter clinical trial studies in the near future.

Fungal metabolites are a rich source of new and potentially bioactive agents with diverse structures, and the enterprise of screening these metabolites for new leads to design and create more active and more specific medical agents will continue as an important avenue toward new drug discovery.

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Commercial Production and Biosynthesis of Fungal Antibiotics: An Overview

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1 INTRODUCTION

The use of antibiotics has had a dramatic effect on the practice of medicine, the pharmaceutical industry, and microbiology. Prior to the discovery of antibiotics, the treatment of infectious diseases was empirical. Various types of antimicrobial agents, including extract of plants, fungi, and lichens have been employed for thousands of years in primitive populations without any scientific knowledge of what the active compounds were. However, the seminal work of Joseph Lister, Louis Pasteur, Robert Koch, and others, identifying microbes as agents of disease and devising means for avoiding infections by the use of disinfectants and antiseptics, made possible rational approaches for treatment of infectious diseases. True antimicrobial therapy became available only in the 1930s with the discovery of the sulfonamides by Gerhardt Domagk. Subsequently, those synthetic agents were found to be competitive inhibitors of the enzymatic incorporation of *p*-aminobenzoic acid into the folic acid biosynthetic pathway. Thus, the sulfonamides became the first targets of early attempts for rational drug modification. Surprisingly, no infectious disease has been eliminated by the use of antibiotics. Thus, most of the bacteria that caused human suffering prior to 1950 are still causing illness, and we have come to the woeful realization that the use of antibiotics has even contributed to the recent phenomenon of emerging infections.

2 COMMERCIAL PRODUCTION

Antibiotics played a key role in the development of the modern pharmaceutical industry. Thus, the discovery of penicillin, and thereafter streptomycin, tetracycline, gentamicin, amphotericin, erythromycin, and other therapeutic compounds from microbes helped transform the traditional chemically based pharmaceutical business into one in which natural products are produced by fermentation processes. The well-known pharmaceutical companies such as Squibb, Merck, Lederle, Gist-Brocades, Antibióticos, and Eli Lilly have a long history, but there is little doubt that a decisive event in their evolution was the discovery that low molecular weight products of microbes have potent antibacterial activity. The introduction of fermentation methods to the industry was also a milestone; the recognition that microbes could be employed as biosynthetic factories on a large scale to generate not only antibiotics, but also a variety of useful products such as enzymes, amino acids, and vitamins was the genesis of the biotechnology industry. The discovery of antibiotics and the maturation of the antibiotic industry illustrate well the marriage of basic and applied science in the development of industrial microbiology.

2.1 Commercial Production of Fungal β-Lactam Antibiotics: Productivity of Penicillin

There are several factors that determine the efficiency, and the economic viability of the fermentation process directed to the production of penicillin: (a) the average productivity of the fermentor by volume and time units, (b) the average efficiency of conversion from substrates to products, and (c) the efficiency of the processes for the recovery and purification of the final product.

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The first two factors depend on the penicillin production capacity with genetically defined industrial strains, as well as the composition of the culture medium. In addition, there are other important variables that affect the productivity of the fermentation such as the transfer of oxygen to the medium, pH, and temperature control of the bioreactor, etc. These conditions factor into controlling the amount of biomass produced that can be grown and maintained during fermentation. The third factor, the efficiency of the process has been improved, since about 90% of the total penicillin recovered in a process taking approximately 15 h. Improvements in the total production have been obtained by improving each one of the above-mentioned factors. Research on the optimization of the fermentation processes and the development of strain improvement programs by pharmaceutical companies has given rise to an spectacular increase in the efficiency of such processes.

2.1.1 Strain Improvement

Improved strains with higher titer of penicillin production are essential for the increase of the productivity by fermentation. Pirt (1983) has estimated that this variable raised the productivity by 16-fold between 1950 and 1975. The improved strains not only produce more penicillin by time unit and energy source, in addition several other characteristics can affect the fermentation process itself (e.g., viscosity of the media, oxygen transfer, etc.). The method consists in the use of mutagenic agents that have random action mechanisms, followed by a previous selection of the mutants. This selection is based directly on the level of antibiotic production, in several processes that influence the production as well as other criteria which influence production or special characteristics and that allows the reduction of fermentation costs (for example the ability to use cheaper substrates, the development of a mycelia structures that facilitates the filtration, etc.). Then the mutants are analyzed by their production at the flask level, and finally by their production at pilot plant scale (Bellgardt 1998).

2.1.2 Mutagenic Agents Used for Strain Improvement

In the classical procedures, two types of mutagenic agents have been employed: physical agents (ultraviolet radiation, gamma rays, x-rays, etc.) and chemical agents [ethyl methanesulfonate (EMS), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG), mustards, nitrous acid, etc.]. The characteristics of the generated mutation will depend on the type of damage produced by the mutagenic agent over the DNA of the microorganism and also on the action of the reparation systems of the microorganism over the mutation. Most of the mutagens employed could produce more than one type of damage over the DNA, but in a different degree: the ultraviolet radiation generally induce the formation of pirimidine dimmers, the ionizing radiation produces a high degree of breakdowns in the chromosome and the EMS and the NTG are alkylating agents. Thus, each mutagen gives rise to a characteristic spectrum of mutants (Auerbach 1976) and it is impossible to predict what kind of mutation is required to improve the production of antibiotics. By this reason several combinations of mutagenic agents and several dosages of them are used with the aim to generate a wide range of mutants to be studied. The susceptibility of one microorganism to be mutated is determined genetically, though the cellular environment can also affect it. Agents and techniques can amplify the mutability of one strain before, during, or after the mutation process: (a) the growth of fungi on a rich medium, immediately after the mutation process, increases the frequency of mutants, (b) retaining mutated spores suspensions without spreading them over the selection media can facilitate the repair process and decreases the total number of mutants, (c) some chemical agents, as the 8-methoxypsoralen (8 MOP) and the acriflavine, reduce the lethal and mutagenic effects of the ultraviolet radiation present before or during irradiation, but induce the opposite effect if they are added after the treatment, (d) caffeine is an inhibiting agent of scission repair, also increasing the cellular mutability.

The protoplast fusion technique has also been used successfully in fungi. It requires obtaining protoplasts (through the use of lytic enzymes that degrade the cell wall of the fungi), and their fusion with concentrations of 40–60% polyethylene glycol and the regeneration on a medium supplemented with an osmotic stabilizator (Anné and Peberdy 1976). It is possible by using this technique to perform crosses between strains that have high level of production and those having low growth rate and strains with low production and a vigorous growth, to obtain strains with the best characteristic of the parental strains. For example, this technique has been used for the recombination between different species of *Cephalosporium* (Hamlyn and Ball 1979) and *Penicillium* (Tahoun 1993).

2.1.3 Selection of Mutants

After the mutagenesis, the process of mutants selection must be carried out, as a previous step for the evaluation of their production. This previous selection can be performed by several procedures (Chang and Elander 1979). Some of these procedures will be described later.

a. Selection Based on the Antibacterial Activity of the Mutants. This consists of the use of growth inhibition assays against bacterial strains sensitive to the product whose activity is to be assayed (in the case of penicillin the sensitive strains will be *Micrococcus luteus* ATCC 93341, *Bacillus subtilis* ATCC 6633, or *Alcaligenes faecalis* ATCC 8750, and in the case of cephalosporin *Escherichia coli* ESS 22-31).

b. Selection of Mutants Resistant to Amino Acid Analogs. Certain amino acids are closely involved in the beta-lactam antibiotic biosynthesis: most notably is the involvement of three amino acids as direct precursors of the β-lactam antibiotic biosynthesis (alpha-aminoadipic acid,

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cysteine, and valine) (see Figure 1). In 1979, Chang and Elander isolated several mutants of *A. chrysogenum* resistant to several amino acid analogs, and also found that the isolation of mutants resistant to certain analogs of methyonine or lysine give rise to a higher frequency of cephalosporin C overproducer strains compared with random selection itself (Chand and Elander 1979).

c. Selection of Mutants Resistant to Polyene Antibiotics. In 1975, it was described that some strains of *Cephaloporium acremonium* and *C. polyaleurum* resistant to the action of certain polyene antibiotics, such as the nystatin produce large amounts of cephalosporin C. This is probably due to the generation of permeability changes on the cell membrane. In 1977, Luengo and coworkers described a

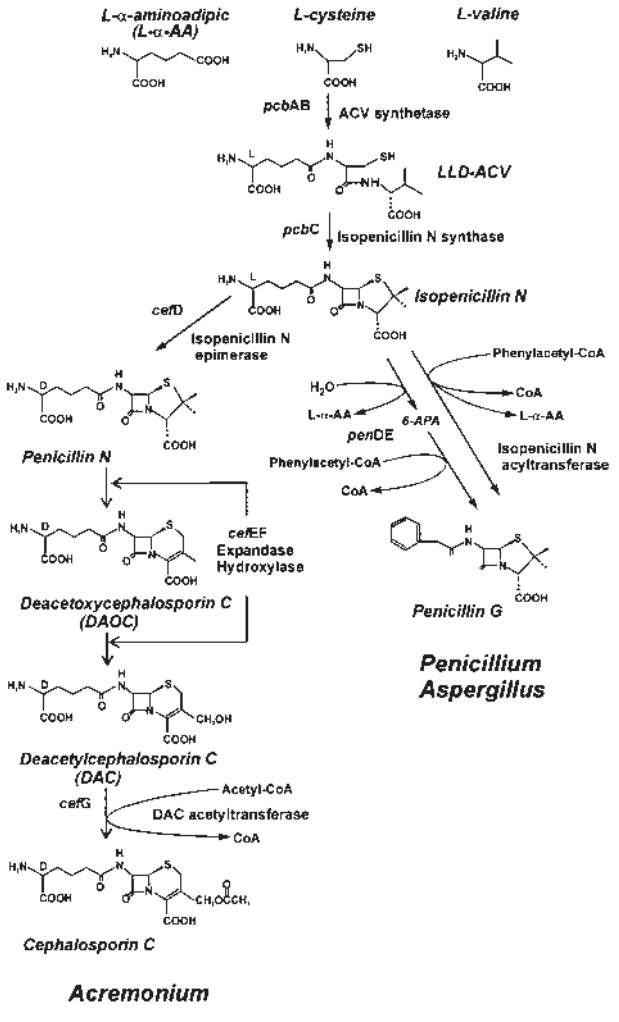

Figure 1 Biosynthetic pathways of penicillin G and cephalosporin C indicating the enzymes catalyzing each reaction and the genes that encode them. LLD-ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. IPN, isopenicillin N. DAOC, deacetoxycephalosporin C. DAC, deacetyl-cephalosporin C.

similar mechanism to select mutants of *Penicillium chrysogenum* with an increased ability to produce penicillin (Luengo et al. 1977).

d. Selection of Mutants Resistant to the Phenylacetic Acid. This acid is known to be a precursor in the biosynthesis of penicillin G, but at high concentrations it is also an inhibitor of the fungal growth. It has been shown that strains which are able to resist elevated concentrations of acid are able to produce more penicillin (Barrios-González et al. 1993).

e. Selection of Mutants Sensitive to the Action of Antimetabolites or Growth Inhibitors. This technique is based on the discovery that the strains of *A. chrysogenum* with increased production of cephalosporin C are usually more sensitive to certain growth inhibitors, as the acriflavine and the *p*-fluorophenylalanine, than the original strains (Chang and Elander 1979). Nuesh and coworkers described in 1976 the existence of a strain of *A. chrysogenum* sensitive to selenomethionine that presents an elevated production of cephalosporin C (probably due to an increased ability to transport methionine) (Nuesch et al. 1976).

f. Selection of Mutants Resistant to Heavy Metal Ions. Some heavy metals, as Hg^{++}, Cu^{++}, and other related ions form complexes with β-lactam antibiotics (Chang and Elander 1979). Thus, mutants resistant to high concentrations of such metal ions could acquire this ability by the increase in the antibiotic production (assumedly detoxifying the ions). The isolation of *C. acremonium* mutants which overproduced cephalosporin when they were selected by resistance to phenylmercuric acetate, Cu^{++}, chromate, Mn^{++}, and Hg^{++} has been described by Lewandowska and Paszewski (1987).

g. Selection of Auxotrophs Followed by Reversion. With this technique a part of the branched biosynthetic pathway is blocked to channel the metabolic flux to the remaining part. This method has been used successfully to increase the production of cephalosporin in *A. chrysogenum* (Treichler et al. 1979) and also penicillin production in *P. chrysogenum* (Casqueiro et al. 1999).

However, independent of the choice used in the selecting mutants, it must be taken in account that the correlation between the ability to produce an antibiotic in the plate vs. a flask or fermentation tank could be very low. Thus, it has been showed that only 10% of the *P. chrysogenum* strains selected by increased production at the plate level showed such an increase in flasks (Ball and McGonagle 1978). In addition, newly selected mutants will have a new phenotype that usually requires new optimization of fermentation conditions in order to get maximum production.

2.1.4 Process Improvement

Fermentor design and media composition have not changed dramatically since the late 1960s and what has changed is our

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growing understanding of the requirements of the fungi to produce more β-lactam antibiotics. This enables us to create better growth conditions for antibiotic production. This requires advanced instrumentation to measure and control key parameters, and this has been the main development of the process since the 1960s until today.

In addition to the well-known parameters such as temperature, pH, dissolved oxygen, inorganic nitrogen, reducing sugars, and biomass that are routinely measured, it is known that many of these parameters are interdependent. For example, the concentration of reducing sugars will influence the growth rate and the biomass concentration. In regards to the fermentation process itself, the preferred mode is still fed-batch that allows for a far more accurate control compared to simple batch fermentation or continuous culture (Patnaik 2001).

Finally, the recovery and purification of the product are the other aspects of the process that can be improved. Thus, in the recovery–purification process, there are several steps such as broth filtration, extraction, extract purification, crystallization, filtration of crystals, and crystal washing and drying. However, it appears that only the extraction step has been optimized, taking into account the following aspects: (a) use of only one extraction step to obtain a sufficient pure final product, (b) the extraction of the whole broth allowing the omission of the broth filtration step, and (c) the use of solvents to extract the stable penicillin from aqueous solution and acid pH because of the risk of primary and quaternary amine degradation (Diender et al. 2002).

3 BIOSYNTHESIS OF ANTIBIOTICS

Antibiotics and other secondary metabolites are synthesized in response to physiological stress due to nutrient limitation, e.g., in response to limitation of phosphate or easily assimilated carbon and nitrogen sources (Martín and Liras 1989). Secondary metabolites, accumulated in response to nutrient starvation may serve as biochemical signals that trigger differentiation or as microbial antagonists that inhibit the growth of competing microorganisms (Chater and Bibb 1997).

3.1 Common Reactions to the Penicillins and Cephalosporins Biosynthesis

3.1.1 Formation of the Alpha-aminoadipyl–cysteinyl–valine Tripeptide

Pioneering studies on the biosynthesis of penicillin using cell free systems led to the crucial observation that the cysteine-containing tripeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) was the direct precursor of these antibiotics (Figure 1). The enzyme that catalyzes this function is the ACV synthetase, which requires ATP and ions Mg^{2+} or Mn^{2+} and has several different activities including the substrate recognition, amino acid activation (adenylation), condensation, epimerization of the L-valine to the D configuration and the cleavage and release of the final tripeptide. The ACV synthetase has three domains with sequences very conserved between them, and separated by regions of low similarity (Gutiérrez et al. 1991). These domains, range from 500 to 1000 amino acids and appear to correspond to the activation center for each precursor amino acid, suggesting a similar organization to the adenylate forming enzymes and to other peptide forming multienzymes (Kleinkauf and von Döhren 1990). Thus, the ACV synthetase contains regions similar to that defined as consensus for the phosphopantetheine binding sequence; this moiety keeps the intermediates bound to the enzyme and translocates these intermediates to other domains (MacCabe et al. 1991). The ACV synthetase also has a thioesterase consensus sequence which should hydrolyze the bond between the enzyme and the tripeptide. Finally, ACV synthetase has between 404 and 425 kDa depending on the microorganism, and is encoded by the *pcb*AB gene which has been cloned from different microorganisms [for a review see Turgay et al. (1992)].

3.1.2 Cyclation of the ACV Tripeptide and Formation of the Isopenicillin N

The ACV tripeptide is cyclized to form isopenicillin N (IPN), an intermediate which contains an L-α-aminoadipyl side chain attached to the *penam* nucleus (Figure 1) by the IPN synthase or cyclase which is encoded by the *pcb*C gene. The IPN synthase of *P. chrysogenum* shows a molecular mass of 39,000 Da. It requires dithiothreitol (DTT) for the activity and is stimulated by ascorbate and Fe^{2+} ions (usual cofactors for oxygenases). The enzymatic reaction requires O_2 and is stimulated by the concentration of the dissolved oxygen in the reaction. In fact, the cyclization occurs by the elimination, mediated by oxygen of four hydrogen atoms from the tripeptide molecule (Aharonowitz et al. 1992).

3.1.3 The Last Step in the Penicillin Biosynthesis: Conversion of IPN into Penicillin G

The α-aminoadipyl side chain is exchanged for phenylacetic acid in penicillin-producing fungi but not in cephalosporin producers. This reaction is catalyzed by an isopenicillin N acyltransferase (IAT) found in extracts of *P. chrysogenum* and *Aspergillus nidulans* (but not, interestingly, in *C. acremonium*) (syn. *Acremonium chrysogenum*). The enzyme shows an optimal pH between 8 and 8.2, and optimal temperature of 25°C. Purified preparation of the acyltransferase shows, after electrophoretic analysis, three protein bands of 40, 29, and 11 kDa. The 40 kDa protein is a heterodimer composed of the two subunits of 11 and 29 kDa. The small subunit corresponds to the amino-terminal end of the protein, which is first synthesized as the 40 kDa protein, and latter is processed into the two subunits (11 and 29 kDa). The acyltransferase protein is encoded by the *pen*DE gene which has been cloned from *P. chrysogenum* (Barredo et al. 1989) and *A. nidulans* (Montenegro et al. 1990).

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3.1.4 Specific Reactions for the Cephalosporins Biosynthesis

Isopenicillin N is converted to penicillin N in cephalosporin producing microorganisms by an IPN epimerase that isomerizes the L-α-aminoadipyl side chain to the D configuration. This enzyme does not require Fe^{2+}, ascorbic acid or ATP, and the activity seems to be stimulated by pyridoxal-5-phosphate.

Penicillin N is transformed into deacetoxycephalosporin C by the deacetoxycephalosporin C synthase (the so-called ring-expanding enzyme or expandase). This enzyme converts the five-membered thiazolidine ring of penicillins into the six-membered dihydrothiazine ring of cephalosporins and requires Fe^{2+} and 2-ketoglutarate. Its activity is strongly stimulated by ascorbic acid and to a lesser extent by ATP, both well-known effectors of 2-ketoglutarate-dependent dioxygenases. The enzyme from *C. acremonium* has a molecular weight of 31 kDa (Kupka et al. 1983). It expands the β-lactam ring of penicillin N but does not accept the isomer IPN, penicillin G, or 6-APA as substrates. Deacetoxycephalosporin C is hydroxylated to deacetylcephalosporin C by another 2-ketoglutarate dependent dioxygenase. This activity catalyzes the incorporation of an oxygen atom from O_2 into the exocyclic methyl group of deacetoxycephalosporin C and is also stimulated by 2-ketoglutarate, ascorbate, DTT, and Fe^{2+}. The deacetoxycephalosporin C synthase and deacetoxycephalosporin C hydroxylase activities from *C. acremonium* are located on a single protein of approximately 36 kDa molecular mass which is encoded by the *cef*EF gene (Samson et al. 1987).

Acetylation of deacetylcephalosporin C to cephalosporin C is the terminal reaction in cephalosporin-producing fungi (Figure 1). The DAC-acetyltransferase was purified to near homogeneity and its amino-terminal end matched perfectly the amino acid sequence deduced from the gene. The purified protein has a molecular mass of 50 kDa; it is encoded by the *cef*G gene, and is not processed into subunits (Gutiérrez et al. 1992).

3.2 Clustering of Genes for the Biosynthesis of β-Lactam Antibiotics

The cloning of the *pcb*C gene of *A. chrysogenum* (Samson et al. 1985), and complementation studies in *P. chrysogenum* using nonpenicillin producing mutants of this fungus led to the conclusion that the adjacent regions to the *pcb*C gene complemented the lack of penicillin production in these mutants. The nucleotide sequence of such regions revealed the presence of the other two penicillin biosynthetic genes (*pcb*AB and *pen*DE) (Díez et al. 1989). Thus, it was concluded that the three structural genes for the penicillin biosynthesis were clustered in the same genomic region of *P. chrysogenum* (Díez et al. 1990) and also in *A. nidulans*

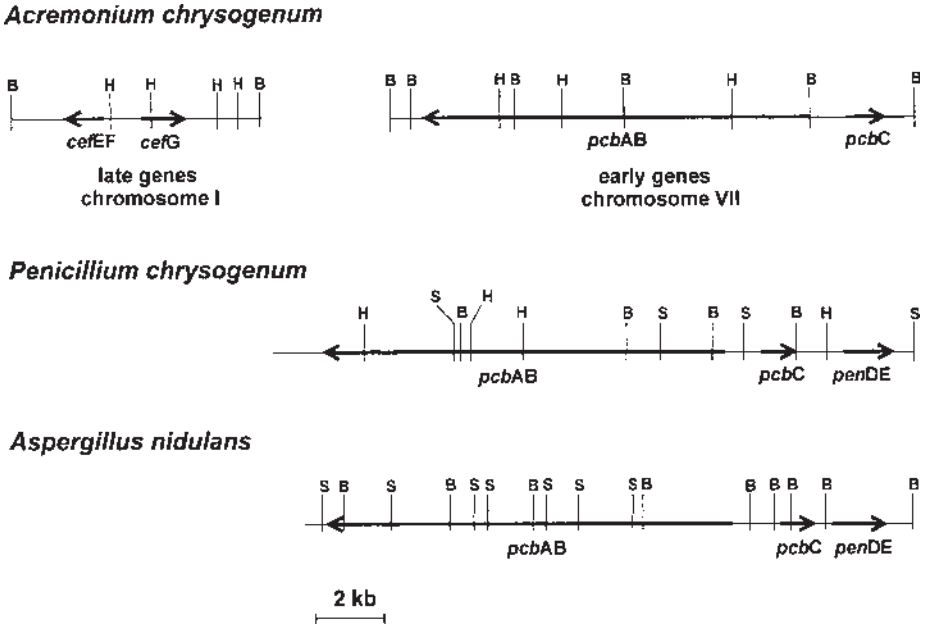

Figure 2 Organization in clusters of the genes involved in β-lactam antibiotics biosynthesis in different filamentous fungi. Note that the genes involved in the biosynthesis of cephalosporin C in *A. chrysogenum* are organized in two clusters (early cluster and late cluster) located in different chromosomes. B, *Bam*HI; K, *Kpn*I; S, *Sal*I; A, *Apa*I; H, *Hin*dIII.

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(MacCabe et al. 1991) (Figure 2). In *A. chrysogenum* the situation was found to be slightly different, the *pcb*AB and *pcb*C genes are linked on chromosome VI in the 394-4 strain (Skatrud and Queener 1989) or chromosome VII in the C10 strain (Gutiérrez et al. 1999b) (Figure 2) forming the so-called "early cluster," whereas *cef*EF and *cef*G genes have been mapped on chromosome II in the 394-4 strain (Skatrud and Queener 1989) or chromosome I in the C10 strain (Gutiérrez et al. 1992) forming the so-called "late cluster" of genes for the cephalosporin C biosynthesis. An intriguing question is what possible advantages are there for the clustering of pathway genes? Certainly clustering facilitates coordinate expression of the penicillin or cephalosporin genes. Furthermore, clustering creates opportunities for the pathways to be transferred in a horizontal manner. In fact, heterologous expression of a fungal penicillin pathway has been demonstrated using a cosmid clone from *P. chrysogenum* carrying the *pcb*AB, *pcb*C, and *pen*DE genes (Smith et al. 1990). From an evolutionary viewpoint, one advantage of pathway gene clustering is that it could increase the probability that pathway genes are transferred as a unit during sexual reproduction or parasexual recombination. A major difference in these two types of genetic exchange is the presence of meiotic crossing over in sexual recombination. Many fungi that contain dispensable pathway gene clusters appear to lack a sexual cycle, and parasexual processes are thought to play important roles in the genetics of these fungi (Saupe et al. 1996).

3.3 Application of the Genetic Engineering to the Production of β-Lactam Antibiotics in Fungi

The use of genetic engineering techniques has given a new perspective in the development of strains producing higher level of antibiotics. They have shown in detail, the nature of the changes originated during the random mutagenesis processes as well as the specific location of such changes in the genome. These data will significantly facilitate, without doubt, future attempts to strain improvement, allowing alterations that should increase the precision of the mutagenesis techniques and effects.

Once almost all the structural genes responsible for the antibiotic biosynthesis were cloned, the first approaches to improve production strains were based on a very simple premise. If production depends on the expression of certain genes, changes, which are able to increase expression in these key genes, should end up being translated as an increase in the biosynthesis down the line. For the development of this kind of strategies it was essential to develop more efficient techniques to transform filamentous fungi: first with *A. nidulans* (Yelton et al. 1984) and later in several other fungal species, such as *C. acremonium* (Peñalva et al. 1985) and *P. chrysogenum* (Cantoral et al. 1987). In all the cases, the methods used were based on modifications of the method developed for *Saccharomyces cerevisiae* (Hinnen et al. 1978) which has three fundamental steps: protoplasts formation from mycelium, DNA transformation of protoplasts, and their regeneration on selective media.

3.3.1 Increasing Copy Number of Genes Involved in the Production of β-Lactam Antibiotics

The first successful application of this technique was with the *cef*EF gene of *C. acremonium* (Skatrud et al. 1989). The amplification of the gene gave rise to an increase in the activity of this protein, a decrease in the amount of accumulated penicillin N, and an increase in the production of cephalosporin C in the studied strain.

In *P. chrysogenum* the introduction of additional copies of the *pcb*C gene in the strain Wisconsin 54-1255 was not able to demonstrate a significant increase in the production of penicillin G. However, the production was accelerated between the 30 and 80 h period of synthesis. This may be explained because IPN synthase should not be a bottleneck in the penicillin biosynthetic pathway. Theoretically, the increase in the enzymatic activity should not be enough to increase the penicillin production. In addition, by utilizing an integrative plasmids for transformation that depends on the integration *locus* of the fungal genome, specific expression of the chosen gene can be affected.

A related experiment was performed utilizing the *pcb*C gene in a wild isolate of *C. acremonium* as well as the mutant N2 and similar results were obtained. Some of the N2 transformants recover the IPN synthase activity and antibiotic production and in the wild strain only some transformants showed increased levels of antibiotics were found (Skatrud et al. 1989). This suggests that the *pcb*C enzyme does not act as a limiting step in the cephalosporin C biosynthesis, at least in those strains.

The *pen*DE gene of *P. chrysogenum* has also been used for this type of study. In this case, a fragment of 5.1 kb containing the *pcb*C and *pen*DE genes was used for the transformation. The average value of penicillin G production was found to be significantly higher than the value of production of those transformants obtained with the control plasmid without insert. The two transformants with the highest production showed an increase of 40% with respect to the production in the transformants used as control, though the variability between the transformants was very high (Veenstra et al. 1991).

3.3.2 Analysis of the Industrial Strains

In *P. chrysogenum* NRRL 1951, *P. chrysogenum* Wisconsin 54-1255, and *P. notatum* ATCC 9478 (Fleming's isolate) there is only one copy of the biosynthetic genes present. However, with industrial strains of *P. chrysogenum*, such as AS-P-78 or P2, a 106.5 kb amplified region was found that contains the three structural genes needed for the penicillin biosynthesis. This amplification results in multiple copies of this region (5–6 copies in the strain AS-P-78 and 6–7 in the case of P2) repeated in tandem (Fierro et al. 1995). One interesting observation was that in the extreme ends of

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the amplified regions, as well as in the junction between the different copies, the same hexanucleotide sequence was found: TTTACA (this also appears surrounding the only copy of this region that is present in *P. chrysogenum* NRRL 1951 and *P. notatum* ATCC 9478) (Figure 3).

In other high productivity industrial strains such as *P. chrysogenum* E1, amplification has also been discovered, although the size of the amplified region is only 57.9 kb. The region contains 12–14 copies of tandem repeats which include the biosynthetic genes. The extremes of the amplified region and the junction between the different copies also contain the same sequence TTTACA described previously or its reverse complementary (TGTAAA) (Figure 3).

The nonproducer mutants *P. chrysogenum npe*10, Bb-1/125, Bb-1/168, and Bb-1/759 (which lack the three penicillin structural biosynthetic genes) showed a 57.9 kb deletion that corresponds exactly with the DNA fragment which is amplified in the E1 strain. Interestingly, the deletion is located in the same place that the hexanucleotide sequence TTTACA is found (Fierro et al. 1995; 1996) (Figure 3).

It has been postulated by Lein (1986) that the powerful mutagenic treatments used with the industrial strains have, on many occasions, resulted in a chromosome instability phenomena. This instability could explain chromosome duplications observed in *A. nidulans* (Sexton and Roper 1984) or to gene amplification phenomena (Burr et al. 1982). In *P. chrysogenum* the amplification in tandem is similar to the one observed in some cases of *S. cerevisiae* (Walton et al. 1986). In this microorganism it has been described that the amplification could give rise to the formation of large molecules of chromosomal DNA while in *P. chrysogenum* the amplification is maintained inside the chromosome forever.

These results could begin to explain the mechanism of gene amplification produced in the industrial strains. The recombination at the site of the conserved hexanucleotide sequence, found at the extremes of the amplified regions on *P. chrysogenum* industrial strains, could be a possible model to explain the formation of the tandem repetitions that appear after the random mutagenesis of *P. chrysogenum* strains. The sequence TTTACA, and its reverse complement, may act as sites for specific recombination at high frequency after nitrosoguanidine treatment. These recombination processes could be a part of a system of reparation similar to the one used by *E. coli* to repair damaged DNA fragments (Walker 1985). Such systems seem to be present in yeast and filamentous fungi and their expression should be induced after the application of the mutagenic treatments (Bainbridge 1981).

3.4 Regulation of β-Lactam Antibiotic Biosynthesis in Fungi

3.4.1 Carbon Catabolite Regulation

a. Carbon Catabolite Regulation of the Penicillin Biosynthesis. Penicillin biosynthesis in *P. chrysogenum* is

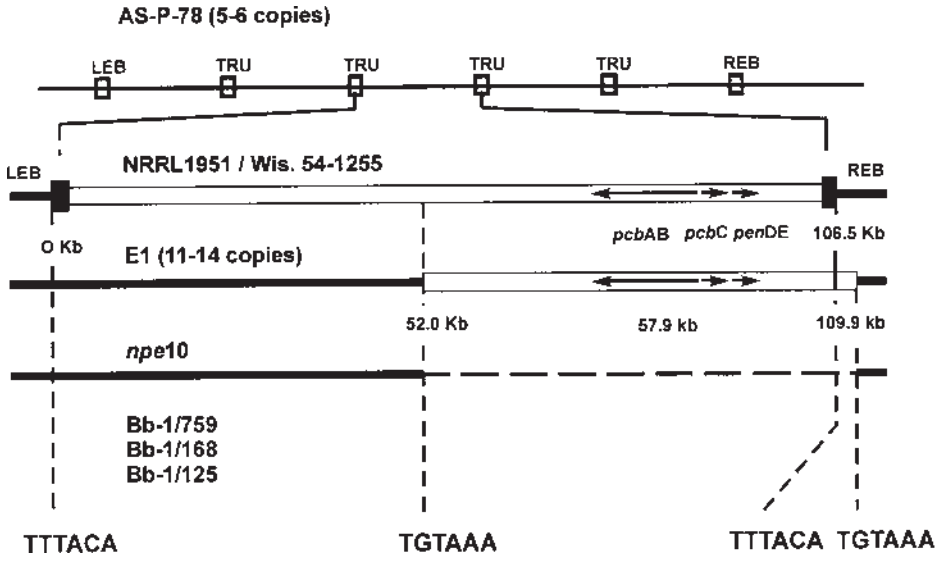

Figure 3 Schematic representation of the amplified regions of *P. chrysogenum* AS-P-78 (106.5 kb) and *P. chrysogenum* E1 (57.9 kb). The left-end border (LEB) and right-end border (REB) fragments are indicated by solid boxes. The arrangement of the five tandem repeats (TRUs) in strain AS-P-78 is indicated in the upper part of the figure. The deleted region in the strains npe10, Bb-1/759, Bb-1/168, and Bb-1/125 of *P. chrysogenum* is indicated in the lower part of the figure by a discontinuous line. In the bottom of the figure the hexanucleotide sequences found in the borders of the amplified regions and also in the borders of the deleted regions of the mutants are indicated.

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known to be strongly regulated by glucose and sucrose and to a lower extent by other sugars (maltose, fructose, and galactose), but not by lactose (Revilla et al. 1986). Lactose has been traditionally used for penicillin biosynthesis although carbon catabolite regulation in batch cultures may now be avoided by slow-feeding of glucose to the fermentors. Penicillin biosynthesis in *A. nidulans* is also repressed by sucrose and to a lower extent by glucose although the mechanism of sucrose control of penicillin gene expression in *A. nidulans* appears to be different from that of *P. chrysogenum* (see below). Interestingly, penicillin biosynthesis in *P. chrysogenum* is also repressed by 2-deoxyglucose (Revilla 1983). This glucose analog is phosphorylated to 2-deoxyglucose-6-phosphate but appears not to be metabolized further in the glycolysis. This may suggest that signal transduction leading to glucose repression of penicillin biosynthesis genes proceeds by modification (perhaps phosphorylation) of a penicillin regulatory protein exerted by glucose or a phosphorylated derivative of this sugar (or its analogs). High glucose concentrations prevent formation of δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (the first intermediate of the pathway) and depress IPN synthase and (only to a low extent) acyl-CoA:IPN acyltransferase (Brakhage et al. 1992). Glucose-grown cultures showed reduced α-aminoadipic acid pools (Hölinger and Kubicek 1989) apparently by stimulating lysine biosynthesis and growth (Revilla et al. 1986).

b. Carbon Source Regulation of Cephalosporin Biosynthesis In C. Acremonium. Early studies provided evidence that cephalosporin production in *C. acremonium* is dependent upon the carbon source used. Carbon sources that provide good growth, e.g., glucose do not support cephalosporin biosynthesis vice versa cephalosporin is produced at higher rates with sugars that support slow growth (Martín and Aharonowtiz 1983). Glucose repression appears to be effect the latter steps of cephalosporin biosynthesis (Zanca and Martín 1983). Zhang and Demain (1989) reported that the level of ACV synthetase in *A. chrysogenum* was not repressed by glucose but they observed an inhibition of the ACV synthetase activity *in vitro*. The ACV synthetase was found to be inhibited by glyceraldehyde 3-phosphate but not by glucose itself (Zhang and Demain 1989). This result is consistent with the observation of the important role of phosphate on glucose repression of penicillin biosynthesis. Few studies have been published on transcriptional regulation by glucose of the cephalosporin biosynthesis genes. However, it is well known that transcription from these promoters is induced by methionine (Velasco et al. 1994). There are three PacC binding sites in the *pcb*AB–*pcb*C intergenic sequence in *A. chrysogenum* (Menne et al. 1994) suggesting that pH regulation of cephalosporin gene expression is also very likely. However, the molecular mechanism of glucose control of cephalosporin biosynthesis at the transcriptional level remains to be elucidated.

3.4.2 Nitrogen Source Regulation

β-Lactam formation by *P. chrysogenum* is strongly regulated by the nitrogen source; ammonium ions show the most potent negative effect. In *A. nidulans* the expression of *pcb*AB and *pcb*C genes is not affected by ammonium. However, ammonium controls the *pcb*C promoter in *P. chrysogenum*. Nitrogen regulation of penicillin formation in *P. chrysogenum* is controlled by a nitrogen regulatory gene (*nre*), this gene encodes a regulatory protein of high identity with the *areA* and *nit-2* encoded proteins from *A. nidulans* and *Neurospora crassa*, respectively, containing a $Cys-X_2-Cys-X_{17}-Cys-X_2-Cys$ type of zinc finger and an adjacent basic region. The NRE protein binds to the intergenic *pcb*AB–*pcb*C region of *P. chrysogenum* which contains two GATA core elements (Wiame et al. 1985).

3.4.3 Regulation by Lysine

Lysine biosynthesis involves L-α-aminoadipic acid (AAA), a precursor of the β-lactam antibiotics, as an intermediate. L-α-aminoadipic acid stimulates penicillin biosynthesis in the absence of added lysine. Since, penicillin G and lysine are products of a branched biosynthetic pathway, an excess of lysine limits production of the common intermediate, AAA, thus interfering with penicillin biosynthesis. A direct correlation between penicillin titer and intracellular AAA in a medium supporting penicillin production has been observed and no other amino acid showed such a correlation. In both growing and resting cells, AAA addition stimulated penicillin production but Cys or Val supplementation did not. The AAA pool is thus one of the limiting factors in the production of ACV and IPN in *P. chrysogenum*. The AAA-reductase appears to be important in relation to the flux from AAA to penicillin in *P. chrysogenum*. The enzyme from three different producers showed decreased affinity to AAA as penicillin production ability increased among the three strains. The addition of 0.1 mM of lysine to *A. nidulans* repressed the expression of the genes encoding ACVS and IPNS proteins. Lysine inhibited penicillin production but not growth, and the effects were not reversed by 1 mM DL-AAA, but this lack of reversion could be due to AAA-transportation problems inside the cell (Bañuelos et al. 2000).

3.4.4 Regulation by Glutamate and Glutamine

Glutamate may have an inductor effect on penicillin formation in *P. chrysogenum*. Glutamate is the most prominent amino acid in the intracellular pool of *P. chrysogenum* and its concentration increases just before penicillin production begins. Resting cell production of penicillin is suppressed by glutamine and all neutral amino acids but not by acidic or basic amino acids. The mechanism postulated is that the AAA portion of the ACV is used to transport neutral amino acids into the cell, forming an intracellular dipeptide (AAA–neutral amino acid), which would reduce the amount of AAA available for penicillin

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biosynthesis. The glutathione showed the same effect as AAA, preventing the inhibitory effect of the neutral amino acids over penicillin production (Lara et al. 1982).

3.4.5 Regulation by PH

Penicillin production is five-fold greater at pH 8.1 than at neutrality in *A. nidulans*. It has been proposed that the *pacC* gene controls the pH effect on penicillin formation. Thus, when it is mutated, production is the same at neutrality than at alkaline pH. The pH control might be related to carbon source repression, since repression of cyclase as measured by mRNA formation is lessened by mutating *pacC*. Recent evidence shows that, in *P. chrysogenum*, repression of the penicillin biosynthetic genes is only partially reversed by alkaline pH values (Espeso and Peñalva 1992; Gutiérrez et al. 1999a).

4 CONCLUSIONS

The role of antibiotics and other secondary metabolites in nature has been a subject of intense discussion for many years. Antibiotics may be antagonist agents to combat bacteria and other microorganisms or molecules that trigger physiological or morphological differentiation. Antibiotics also played a key role in the development of the modern pharmaceutical industry. Thus, the discovery of penicillin, and thereafter other therapeutic compounds from microbes helped the transformation of the pharmaceutical business into one in which natural products are produced by fermentation processes. In the process of antibiotics production itself, there are several factors that determine the efficiency, and by the way the economic viability of the fermentation process directed to the production of this kind of compounds. Thus, the research over the optimization of the fermentation processes and the development of strain improvement programs by the pharmaceutical companies has given rise to a spectacular increase in the rentability of such processes. Obtaining improved strains with higher titer of penicillin production has been essential for the increase of the productivity by fermentation. These improved strains not only produce more amount of penicillin by time unit and energy source, in addition several other characteristics can be beneficiate that affect to the fermentation process itself, as they are the viscosity of the media, oxygen transfer, etc. On the other hand, since the beginning of the industrial production of penicillin, the more remarkable advance in this field has been our growing understanding of the needs of the fungi to produce more antibiotic, allowing us to create better conditions for antibiotic production and also to develop advanced instrumentation to measure and control key parameters. The genes involved in the biosynthesis of these antibiotics appear to be located in groups "clustered" on the chromosome and actually many clusters of genes involved in the biosynthesis of different antibiotics have been isolated. The use of genetic engineering techniques has allowed us to give a new perspective in the development of strains with higher level of antibiotics production, but also it has served to know in detail the nature of the changes originated during the random mutagenesis processes and the specific location of such changes in the genome of the microorganism. Thus, one of the first approaches using these kinds of techniques has been the increase of gene copy number with the aim to increase the production of one particular product. Interestingly, these phenomena of gene amplification are the same that happens in many of the industrial strains, though in these cases they are the result of the application of classical mutagenesis techniques. Taking into account the considerations discussed earlier the future of commercial antibiotic production must be directed to a better characterization of the industrial strains by the use of genomics and also by proteomic approaches. Today almost all the genes involved in the biosynthesis of β-lactam antibiotics in fungi have been cloned and characterized, the aspects about the biosynthesis of such antibiotics that are known in a lesser extend are the regulatory genes or regulatory pathways that control the expression of the structural genes involved in the β-lactam antibiotics biosynthesis. Although some of the regulatory genes have been cloned and studied "*in vitro*" as they are the genes involved in the pH, carbon, and nitrogen regulation (Brakhage 1998), it seems essential to perform "*in vivo*" studies to know the effect of the proteins encoded by such regulatory genes over the antibiotic production in the fermentation conditions. Thus, the actual development of the genomic and proteomic techniques allows their use for such studies. On the other hand, for the future of the commercial production of antibiotics it will also be very important to obtain higher production strains which should be able to use the substrates more efficiently and also the development of more efficient techniques of fermentation and product purification.

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Molecular Biology of *Trichoderma* and Biotechnological Applications

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1 INTRODUCTION

Trichoderma spp. comprise common soil Hyphomycetes found in all climate zones ranging from Antarctica to the tropics (Domsch et al. 1980). Characteristic of the genus *Trichoderma* is the ability to effectively degrade a range of natural polymers, especially cellulose and hemicellulose, which also provides foundation for the commercial uses of *Trichoderma*. The taxonomy of the genus *Trichoderma* has been and still is under some dispute, however, the basis being on the work of Rifai (1969) assigning the *Trichoderma* strains to nine species aggregates differentiated primarily by patterns of conidiophore branching and conidium morphology. Recent studies have used DNA fingerprinting, restriction fragment length polymorphism and amplification, and ITS sequencing to further clarify the taxonomy of *Trichoderma* (revieved in Gams and Bissett 1998; Lieckfeldt et al. 1998). This analysis has revealed that the industrially most relevant species, *Trichoderma reesei*, is an anamorph of the species *Hypocrea jecorina*. A genetically well-defined lineage of strains originating from the isolate QM6a was classified as *T. reesei* and all current biotechnologically relevant strains are derivatives of QM6a (see Nevalainen et al. 1994), which is a unique situation.

Trichoderma research has not only made important contributions to biotechnology but also contributed to several aspects of basic science. One of the biggest impacts has been in understanding of cellulase enzyme function. The cellulase encoding genes of *T. reesei* were among the very first genes isolated from filamentous fungi (Chen et al. 1987; Penttilä et al. 1986; Shoemaker et al. 1983; Teeri et al. 1983; Teeri et al. 1987). Their sequencing made possible a direct comparison to the *Saccharomyces cerevisiae* gene structures, which led to immediate recognition of some of the differences between these organism groups. The 3-D structures of the cellulase CBHII, and the cellulose binding domain (CBD) of CBHI were the first structures obtained for enzymes attacking polymeric carbohydrate structures, revealing a unique multi-domain structure and active site architecture (Kraulis et al. 1989; Rouvinen et al. 1990) (Figure 1). This field has subsequently expanded enormously. In addition, the best understanding we currently have on molecular mechanisms involved in the regulation of cellulase gene expression has been obtained with *Trichoderma* (reviewed by Kubicek and Penttilä 1998).

On the applied side, the development of *Trichoderma* as a versatile host for enzyme production has been one of the major contributions. *Trichoderma* is used for large-scale enzyme production by many companies world-wide. *Trichoderma* system offers strong inducible promoters and high secretion levels of extracellular proteins (up to 40 g/l reported). It was among the first filamentous fungi to be transformed and together with *Aspergillus*, the fungus used to demonstrate that foreign proteins including multichain molecules such as antibodies (Nyyssönen et al. 1993) can be produced in filamentous fungi. Original driving force for the host strain development was the application potential of the *Trichoderma* enzymes. The xylanase-aided bleaching of pulp and enzymatic "stone washing" of jeans with cellulases are among the examples that demonstrated the viability of specific tailor-made enzyme preparations in large scale

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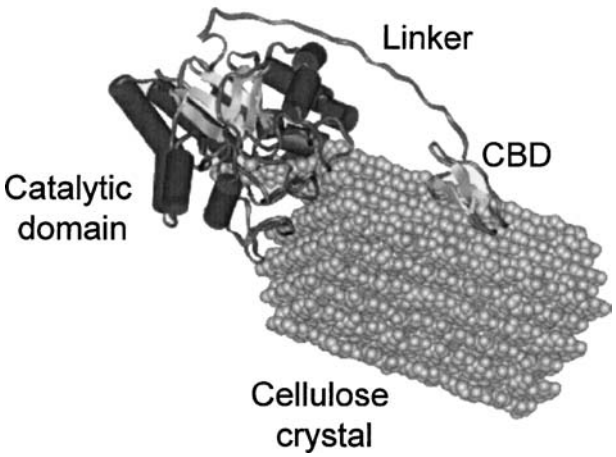

Figure 1 *T. reesei* cellobiohydrolase II, CBHII, attacking crystalline cellulose. The enzyme attaches on the substrate by the cellulose binding domain (CBD). The cellulose chain enters the tunnellike active site where hydrolysis occurs and the disaccharide cellobiose (not seen) is released and liberated from the other side of the active site tunnel. There exists no determined structure for the extended glycosylated linker region connecting the catalytic domain and the CBD. Courtesy of VTT Biotechnology.

industrial processes, discussed in Section "Regulation of cellulase and hemicellulase gene expression." Enzymes from *Trichoderma* have also been expressed in yeast, and the demonstration that improved filtration of beer can be obtained with a recombinant brewer's yeast producing a *Trichoderma* endoglucanase was among the first ones in the field (Penttilä et al. 1987). Since then almost all cloned *T. reesei* genes have been expressed and the enzymes secreted in active form in *S. cerevisiae*. The current renewed interest in biomass utilization for the production of bioethanol and chemicals that requires total hydrolysis of the raw material to fermentable sugars, has further increased the interest in *Trichoderma*. This organism has kept its original position as a producer of one of the most efficient enzyme portfolios for lignocellulose hydrolysis. A comprehensive monograph series on *Trichoderma* has been recently published (Harman and Kubicek 1998), which is recommended to readers interested in *Trichoderma*, or in fungi in general. The current review aims to cover only the most important molecular findings on *Trichoderma* with relevance to biotechnology and discuss more extensively only the most recent literature. We also discuss some newer areas of biotechnological interest, i.e., hydrophobins. The antifungal properties of *Trichoderma* species and their use as biocontrol agents is dealt in this volume in the chapter by C. Kubicek.

2 ENZYMES AND THEIR INDUSTRIAL USE

2.1 General Enzyme Properties

Trichoderma species produce enzymes that hydrolyse efficiently the polymeric carbohydrates cellulose and hemicellulose present in lignocellulosic materials to oligosaccharides and finally to monomeric sugars. Cellulose is composed of long polymers of β-1,4-linked D-glucopyranose units. Cellulose fibers contain amorphous and crystalline regions, the latter being more resistant to hydrolysis. In hemicelluloses, the backbone structure consists of β-1,4-linked D-xylopyranosyl units (xylan) or β-1,4-linked D-mannose and D-glucose units (mannan) with sugar side chains that may be acetylated and/or methylated (reviewed in Tenkanen 1995).

At least three different types of enzymes that act synergistically are required for the hydrolysis of cellulose to glucose: endo (1,4)-β-D-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). Currently, genes encoding two cellobiohydrolases (*cbh1*, *cbh2*), at least five endoglucanases (*egl1*, *egl2*, *egl3*, *egl4*, *egl5*) and at least one extracellular β-glucosidase (*bgl1*) have been reported (reviewed by Koivula et al. 1998; Nevalainen and Nevalainen and Penttilä (2003); Penttilä 1995; Teeri et al. 1992) but the number of enzymes involved in nature in cellulose hydrolysis may still be larger. Due to the chemically more varied structures in hemicelluloses (xylans, mannans, arabinans), the number of enzymes involved in the hydrolysis of the backbone and the side chain sugars is expected to be very large. Representatives of all major activities needed for hydrolysis have been characterised from *T. reesei* including the endo-β-xylanases (*xyn1*, *xyn2*), endomannase (*man1*), β-xylosidase (*bxl1*), acetyl xylan esterase (*axe1*), arabinofuranosidase (*abf1*), α-galactosidases (*agl1*, *agl2*, *agl3*), and α-glucuronidase (*glr1*) (reviewed by Biely and Tenkanen 1998). The biochemical properties as well as their activity towards natural substrates have been extensively studied. Furthermore, many of the *Trichoderma* enzymes have been crystallised. Three-dimensional structures of the *Trichoderma* cellulases include those of ellobiohydrolase I (CBHI; Cel7A) (Divne et al. 1994), CBH II (Cel6A) (Rouvinen et al. 1990), endoglucanase I (EGI; Cel7B) (Kleywegt et al. 1997), and EGIII (Cel12A; Sandgren et al. 2001), and the endoxylanases XYNI and XYNII (Campbell et al. 1995; Törrönen et al. 1994; Törrönen and Rouvinen 1995) and the endomannanase MANI (Sabini et al. 2000).

Almost all cellulases have a multidomain structure with the catalytic domain and the substrate binding domain (Figure 1) separated from each other by a flexible extended linker. This is also true for some of the hemicellulases. The structure of the CBD has also been determined and shows exposed aromatic amino acids that mediate the binding to cellulose surface (Kraulis et al. 1989; reviewed by Linder and Teeri 1996). The structures of the catalytic domains revealed that cellobiohydrolases have a tunnellike active site whereas that of endoglucanases is a more open cleft. This explains the mode of action: cellobiohydrolases cleave the cellulose

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chains only from the chain ends mainly releasing cellobiose (two glucoses) (Figure 1), whereas endoglucanases act in endo-fashion being able to attack the chain from the middle and produce oligosaccharides of various lengths. The hydrolysis of the glycosidic bonds occurs by general acid catalysis with an involvement of two carboxylic amino acids (reviewed by Koivula et al. 1998; Koivula et al. 2002). Like in the CBD, binding of the glucose residues of the cellulose chain into the active site is aided by aromatic amino acids (reviewed by Koivula et al. 1998; Teeri et al. 1992).

A recent addition to the protein portfolio of *T. reesei* is swollenin, SWOI (Saloheimo et al. 2002). Swollenin has a CBD connected via a linker to a core that shows amino acid similarity to plant expansins. Expansins are involved in plant cell wall growth and suggested to disrupt hydrogen bonding between cellulose microfibrils or between cellulose and other cell wall polysaccharides. The SWOI protein has been shown to have no or very low hydrolytic activity but an ability to disrupt the structure of cellulosic fibers. It is possible that such an activity could aid in opening up the complex lignocellulosic structures for the action of the hydrolytic enzymes.

2.2 Protein Engineering for Improved Performance

The structural data on cellulases and hemicellulases have enabled detailed structure–function analysis of the catalytic mechanism and substrate binding and the identification of the amino acids involved in these mechanisms (reviewed e.g., by Koivula et al. 1998). Already, some of these data has proved useful in terms of applications.

Protein engineering of the active site residues in *T. reesei* CBHI resulted in a modified enzyme exhibiting a more alkaline pH optimum (Becker et al. 2001). Based on comparisons of the structures of the *T. reesei* CBHI and a *Humicola insolens* enzyme, modelling excercises had suggested that a histidine residue near the catalytic amino acids could be a reason for the higher pH optimum of the *Humicola* enzyme. This was confirmed by the introduction of a histidine and some additional changes in the active site of *T. reesei* CBHI (Becker et al. 2001). On the other hand, engineering of the thermal stability *T. reesei* -endoxylanase XYNII also resulted in a more alkaline pH optimum (Turunen et al. 2001; Turunen et al. (2002)). This was achieved through several mutations including for instance disulfide bridge formation. There was no essential difference between the specific activities of the mutants and the wild-type XYNII. Furthermore, the effect of mutagenesis of the tryptophan residues in the active site tunnel subsites of the *T. reesei* CBHII have been studied for substrate binding properties and enzyme kinetics (Koivula et al. 1996; Ruohonen et al. 1993). Interestingly, site-specific mutagenesis of the tryptophan 272, which lies at the entrance of the active site tunnel, revealed that this residue has a particular role in crystalline cellulose degradation in forming an additional subsite for sugar chain binding (Koivula et al. 1998).

The presence of the substrate-binding domain is essential for efficient hydrolysis of polymeric substrates. The binding of the CBD of the *T. reesei* CBHI is reversible and occurs by dilution (Linder and Teeri 1996) and that of CBHII can be dissociated from cellulose by a temperature shift (Carrard and Linder 1999). Site-specific mutagenesis has been used to desing a pH-dependent cellulose-binding domain (Linder et al. 1999). Understanding the binding kinetics is of importance for the application of cellulases in e.g., enzymatic hydrolysis of cellulosic biomass to ethanol, requiring effective recycling of the enzymes. Efficient use of all binding sites on the substrate may depend on the types of the substrate-binding domain present on the enzymes and different types; those originating from fungi and bacteria, may improve the overall enzyme coverage on the substrate (Carrard et al. 2000). Another example of domain engineering is the construction of a hybrid endoglucanase having a core domain of *Bacillus subtilis* and the CDB of *Trichoderma viride* (Kim et al. 1998). This protein bound better to microcrystalline cellulose and thus had enchanced hydrolytic activity. In addition, a fusion of the *T. reesei* CBHII CBD that also binds chitin, to an endochitinase of *Trichoderma harzianum* resulted in improved hydrolysis of insoluble chitin (Limón et al. 2001). Substrate binding domains can also be utilized in other type of biotechnological applications, e.g., in coupling of antibody molecules on cellulosic supports (Linder et al. 1998).

2.3 Regulation of Cellulase and Hemicellulase Gene Expression

Expression of the cellulase and hemicellulase genes in *T. reesei* is provoked in the presence of natural polymeric substrates such as cellulose and various hemicelluloses and small oligosaccharides such as cellobiose, xylobiose, lactose, and sophorose, whereas their expression is usually repressed by glucose, the preferred carbon source for growth (reviewed by Kubicek and Penttilä 1998; Nevalainen and Penttilä 2003). The difference in expression levels between the repressed and induced conditions can be several thousand-fold as shown for the *T. reesei* cellulase *cbh1* (Ilmén et al. 1997). The strict regulation of (hemi)cellulase expression is understandable since the production and secretion of large amounts of a number of hydrolytic enzymes, when not needed, would be a waste of energy for the fungus. There also seem to exist overlapping regulatory mechanisms between cellulases and various hemicellulases, which most likely reflects the close existence of these polymers in lignocellulosic material in the nature.

Glucose repression is at least partly mediated by the glucose repressor protein CREI (Ilmén et al. 1996b; Strauss et al. 1995; Takashima et al. 1996). The CRE protein binds to the concensus promoter sequence 5′-SYGGRG via two C_2H_2 zinc fingers. Interestingly, the hypercellylotic mutant RutC-30 of *T. reesei* that produces some cellulase activity on

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glucose, was found to be mutated in the *cre1* gene so that only the N-terminal amino acids were retained and only one of the zinc fingers was present (Ilmén et al. 1996b). Mutations in the *cre1* gene led to expression on glucose to varying extent of the otherwise glucose-repressed genes such as the cellulase and various hemicellulase genes of *T. reesei* (Ilmén et al. 1996b; Margolles-Clark et al. 1997). Furthermore, mutation of the CREI binding sites in the *cbh1* promoter resulted in the expression of the *cbh1* promoter on glucose (Ilmén et al. 1996b). In addition to binding to the sites in the *cbh1* promoter (Ilmén et al. 1997), CREI has been shown to bind regions in the cellobiohydrolase II, *cbh2*, and the endoxylanase, *xyn1*, promoters of *T. reesei* (Strauss et al. (1995); Takashima et al. 1996).

The most potent fungal activator of (hemi)cellulase expression until now described seems to be the *Aspergillus niger* XlnR, which was originally described as a xylanase activator but has been shown to regulate cellulases as well (van Peij et al. 1998). There is a homologue in *T. reesei* (R. Mach, personal communication 2002) but its specific function is not yet known. However, two new genes *ace1* and *ace2* encoding regulatory proteins affecting cellulase and hemicellulase production were recently discovered from *T. reesei*. These were cloned using a yeast-based cloning system, in which an over 1 kb fragment of the *cbh1* promoter was coupled to the yeast TATA box and a reporter gene. Screening of a *T. reesei* expression library in this yeast resulted in the discovery of genes that encoded factors that could simultaneously bind to the *cbh1* promoter and activate expression of the reporter gene in yeast. The ACEI protein carries three Cys2–His_2 type zinc fingers (Saloheimo et al. 2000), and the ACEII protein a zinc binuclear cluster domain (Aro et al. 2001) for DNA binding. *In vitro* binding assays showed that there are eight binding sites for ACEI scattered along the *cbh1* promoter, which all contain the core sequence 5′-AGGCA. ACEII was found to bind *in vitro* to the sequence 5′-GGCTAATAA, which interestingly is similar to the XlnR binding site, however there is no amino acid similarity in these two proteins outside the DNA binding domain. Both ACEI (Aro et al. 2003) and ACEII (Aro et al. 2001) regulate, in addition to cellulases, also endoxylanase expression in *T. reesei*. Suprisingly, despite its activator function in yeast, deletion of *ace1* from the *T. reesei* genome actually increased expression of the main cellulase genes and the xylanase genes *xyn1* and *xyn2*, indicating that its effect is negative (Aro et al. 2003). ACEII on the other hand seems to be an activator since *ace2* deletion strain had lowered expression of the genes investigated. In this case, the interesting observation was that this effect was only seen in cellulose-based cultures, and not when cellulase expression was induced with sophorose (Aro et al. 2001). That cellulose and sophorose may provoke expression through different mechanisms is also supported by *cbh1* promoter analyses, which showed that certain promoter variants are inducible by sophorose but not by cellulose (Henrique-Silva et al. 1996; Ilmén et al. 1998).

Deletion analyses of the *T. reesei cbh1* and *cbh2* promoters have suggested the involvement of yet other regulatory factors (Ilmén et al. (1996b); Henrique-Silva et al. 1996; Zeilinger et al. 1998). *In vitro* mobility shift assays and DNA footprinting analysis led to the identification of a CAE element in the *cbh2* promoter. This region is proposed to consist of two partially overlapping regulatory regions, a CCAAT motif binding the HAP complex, and a sequence 5′-GGGTAA, both essential for full induction of the promoter *in vivo* (Zeilinger et al. 1998; Zeilinger et al. (2001)). Deletion of the CCAAT motif reduced sophorose-mediated induction by approximately 20%. The HAP proteins are involved in other organisms in various functions including oxidative phosphorylation on non-fermentable carbon sources. Cellulase expression in relation to mitochondrial functions has been studied in *T. reesei* (Abrahao-Neto et al. 1995). The genes encoding (parts of) the HAP complex have been cloned from *T. reesei* (Zeilinger et al. 2001). As is the case with the other regulatory proteins, the HAP complex may regulate xylanase expression as well (Zeilinger et al. 1996).

The regulation of extracellular enzyme production, i.e., how the expression of the various enzyme classes is modulated to provide the fungus most suitable means to respond to the environmental conditions, is expected to be much more complex than we know of today. However, already now the knowledge we have on inducing and repressing conditions and the regulatory factors involved has given us the possibility to improve industrial protein production by *Trichoderma*.

2.4 Industrial Enzyme Applications

The current main industrial application areas for *Trichoderma* enzymes are in the food-processing, pulp and paper, textile, and feed industries. Other sectors where *Trichoderma* enzymes have found uses are detergent industry, biomass hydrolysis, and some environmental applications (Table 1). Brand names such as Crystalzyme, Econase, Stonenzyme, Cellubrix, Ecopulp, and Pulpzyme reflect the targeted uses of enzyme products. Examples of emerging areas for the application of *Trichoderma* enzymes include for instance their development as antifouling agents to replace biocides in marine antifouling paints. There are several recent reviews concerning the industrial applications of the *Trichoderma* enzymes (Benítez et al. 1998; Buchert et al. 1998; Galante et al. 1998a,b).

Considerable experience in the safe use of genetically modified *T. reesei* strains in industrial-scale processes has been accumulated over the years, indicating that this fungus can serve as a safe host for a variety of gene products. *T. reesei* strains producing cellulases have been evaluated as belonging to Group I (low-risk level) microorganisms (EC Directive 90/219/EEC). Pathogenicity tests carried out with genetically engineered *T. reesei* strains producing modified enzyme profiles, e.g., lacking the main cellobiohydrolase, have shown that modification does not alter their safety status (Harman and Kubicek 1998).

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Table 1 Industrial uses of *Trichoderma* enzymes

Industry sector	Description of product	Function
Food		
Grain starch processing	Cellulase	Decreases viscosity, reduces fouling, increases purity of starch
Grain alcohol production	Cellulase	Decreases viscosity, increases yield, reduces fouling, shortens fermentation time
Brewing	Beta glucanase	Decreases viscosity, speeds up the process, improves filtrability
Fruit and berry processing	Cellulase	Decreases viscosity, reduces clouding
	Compounded cellulase	Improves aroma
	Hemicellulase	
Baking	Cellulase	Improves rheology and baking properties
	Xylanase	
Feed		
Digestion	Hemicellulase	Improves weight gain and feed utilization
	Cellulase	
Pulp and paper		
Biobleaching	Xylanase	Reduces the need of chlorine, removes colour environmentally nonpolluting
Paper recycling	Xylanase	Improves toner removal, better drainage
	Cellulase	
Textile		
Biostoning	Cellulase	Reduces the wear and tear of washing machines, Allows higher garment loading, environmentally safer
	Compounded cellulase	
Biofinishing	Cellulase	Removes mops and surface fuzziness, softens and improves the hand feel and dye affinity
Detergent		
Stain removal	Cellulase	Improves stain removal, environmentally beneficial
Biomass hydrolysis		
Effective biomass Degradation	Cellulase	Reduces viscosity, produces fermentable sugars, Improves processing performance
	Compounded cellulase	
Environment		
Composting and waste treatment	Cellulase	Aids in biomass degradation, Improves compost quality

A large majority of the commercial enzymes sold today have been produced by genetically modified strains that may have been further improved through classical mutagenesis procedures (see Section 3.1).

3 SECRETORY PATHWAY EVENTS AND PROTEIN PRODUCTION

3.1 Protein Production Strategies

Genetic engineering is used for the modification of the native enzyme profiles produced by *Trichoderma* to target particular applications (e.g., Harkki et al. 1991; Karhunen et al. 1993; Mäntylä et al. 1998; Suominen et al. 1993). Table 2 lists some examples on tailoring of suitable enzyme mixtures for specific applications. The strong *T. reesei cbh1* promoter is commonly used to increase the level of a wanted enzyme component and targeted gene inactivation is used to knock out unwanted activities such as the cellulase activity detrimental to cellulose fibers from a product aimed for bleaching. Various genetic engineering strategies used in protein production are described in detail in the chapter by Nevalainen et al. in this volume.

Several heterologous products have been expressed and secreted in *T. reesei* including calf chymosin, antibody Fab fragments and single chain antibodies, interleukin-6, tissue plasminogen activator (tPA), and heterologous fungal

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Table 2 Examples of modification of hydrolase profiles according to specific applications

Process	Enzyme requirement	Modification
Starch processing	High endoglucanase	Increase the copy number of the main endoglucanase *egl1*
Animal feed manufacture	High endoglucanase and hemicellulase	Increase the copy number of the main endoglucanase *egl1* gene and xylanase and mannanase encoding genes
Biomass hydrolysis to fermentable sugars	High endoglucanase, high β-glucosidase	Increase the copy number of the main endoglucanase *egl1* and the*bgl 1* gene
Wine making, extraction of fruit juices	Improvement of aroma, clarification of juice	Increase the copy number of the main endoglucanase *egl1* and xylanase and pectinase encoding genes
Pulp bleaching	High xylanase and low or no cellulases	Increase the copy number of xylanase encoding genes and inactivate the main cellulase gene *cbh1*
Textile processing	Jeans washing, biofinishing,	Increase the copy number of e.g., *egl3*, inactivate *egl2*

proteins such as laccase, chitinase, and acid phospatase (reviewed by Keränen and Penttilä 1995; Penttilä 1998), as well as thermophilic enzymes (see Section "Expression of Heterologous Thermophilic Enzymes"). Secreted yields for heterologous fungal enzymes have reached grams per liter level (Paloheimo et al. 1993), although the levels of some proteins such as lignin peroxidases and certain laccases may remain lower. In particular production of proteins from unrelated species benefit from production as fusions to a well-expressed native protein such as CBHI (the CBD domain is replaced with the foreign protein). The fusion protein can be designed so that the protein of interest is cleaved from the CBHI part by an intracellular Golgi-resident KEX2-type protease. Good yields of antibody Fab fragments and calf chymosin have been obtained in *T. reesei*. However, the levels of many mammalian proteins are still significantly lower when produced in filamentous fungi compered with yields of native fungal proteins, one reason for this being proteolysis.

In order to understand the secretory pathway of filamentous fungi in the hope of eventually being able to improve secretion, the cloning of genes involved in the secretion processes have been started also from *Trichoderma*. Among these an interesting one is RHOIII that is a general signal transduction molecule. Disruption of the RHOIII encoding gene from *T. reesei* did not have any effect on glucose or cellobiose but interestingly, reduced growth and secretion of cellulases on cellulose-containing medium (Vasara et al. 2001). The most important contributions, however, have been made in the field of ER-related events that contribute significantly to the folding of proteins and are believed to be one of the main reasons for low yields of heterologous products (see next section).

3.2 Signalling During Secretion Stress

It is generally believed that one of the major bottlenecks in production of heterologous proteins in any host is their folding in the endoplasmic reticulum (ER). Unfolded proteins accumulate in the ER and induce a signalling pathway, the unfolded protein response (UPR) that upregulates a number of genes including those encoding folding factors such as BiP and PDI (protein disulfide isomerase). The PDI (Saloheimo et al. 1999) and BiP (M. Saloheimo, personal communication 2000) encoding genes have been cloned from *T. reesei*. In addition to Bip, a gene encoding seemingly a cytoplasmic hsp70 protein has been isolated (J. Te'o, personal communication 2000). In *T. reesei*, *bip1* and *pdi1* expression is induced by secretion stress created by addition to the cultures DTT or Brefeldin A that cause defects in folding or secretion (Saloheimo et al. 2002; T. Pakula, personal communication 2000) indicating that the UPR pathway is operational. Also expression of antibody fragments (Saloheimo et al. 1999) and tPA (J. Uusitalo and T. Pakula, personal communication 2000) in *T. reesei* and high levels of native secreted proteins cause upregulation of these folding factors.

The gene encoding the positive regulator of the UPR pathway, *hac1*, was recently cloned from *T. reesei* (Saloheimo et al. 2002). In addition, the kinase encoding gene *ire1* and the phosphatase *ptc2* gene have been isolated

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(M. Valkonen and M. Saloheimo, personal communication 2001). The mechanism of HACI activation was studied in detail. Similarly to the yeast *S. cerevisiae*, an unconventional splicing event (mediated by IREI) is needed for the production of an active form of HACI. In addition to that, a truncation at the 5′ end of the *hac1* mRNA also occurs in *T. reesei* (and *A. nidulans)*, which removes upstream open reading frames (ORF) from the transcript. These ORFs appear to mediate translational control (Saloheimo et al. 2002). Overexpression of this regulator increased expression of *bip1* and *pdi1* in *T. reesei* and most likely also other factors under HACI control. In *S. cerevisiae*, Hac1p controls, in addition to the folding factors, a variety of cellular phenomena related to protein secretion such as proteolysis through the ERAD pathway, and genes involved in glycosylation and in the secretion process itself (Travers et al. 2000). The ER-associated protein degradation pathway (ERAD) eliminates misfolded proteins by their degradation in the cytosol in the 26S proteasome. One gene, *prs12*, a homologue of mouse regulatory subunit 12 of the 26S proteasome has been characterised from *T. reesei* (Goller and Kubicek (1998)) and the proteasome has recently been isolated (S. Hauge, personal communication 2001).

Importantly, overexpression of *T. reesei hac1* in the yeast *S. cerevisiae* increased extracellular production of *Bacillus* α-amylase and the endogenous protein invertase (Valkonen et al. 2002). Also, overexpression of the *A. niger hacA* increased production of a heterologous laccase in *A. niger* several fold as well as the expression of calf chymosin (M. Valkonen and M. Saloheimo, personal communication 2001). On the other hand, the *T. reesei hac1* gene did not have a beneficial effect on expression of the same laccase or chymosin in *T. reesei* (M. Valkonen, personal communication 2001). These data show that improved strains can be obtained by upregulation of the complete UPR pathway. However, the effect may depend on the strain used or the particular foreign protein expressed.

A novel observation has been made when studying *T. reesei* strains treated with DTT or Brefeldin A or producing tPA. While the *hac1* mRNA is shortened and the *pdi1* and *bip1* mRNAs subsequently induced, mRNAs encodind secreted proteins such as cellulases and hemicellulases are reduced significantly (T. Pakula, personal communication 2001). This indicates that there exists a signalling mechanism that down-regulates expression of these genes when the fungus is experiencing problems in the secretory pathway. This type of a mechanism makes sense in particular in organisms that produce large amounts of secreted proteins. The detailed mechanisms of this phenomenon are waiting for further investigations.

3.3 Glycosylation

Studies on the effect of glycosylation on secretion of *T. reesei* cellulases have produced unambiguous results so far. For example, N-glycosylation has shown not to be obligatory for cellulase enzyme activity and secretion, however, the *O*-linked glycosylation seemed necessary for the secretion of two endoglucanases, EGI and EGII (Kubicek et al. 1987). In another study, N-glycosylation has contributed to thermostability of cellulases and their resistance to proteolysis (Merivuori et al. 1985; Wang et al. 1996). Finally, several studies on glycopeptide linkers from other glycosylhydrolases have indicated the linker plays some role in thermostability and secretion (see references in Nevalainen et al. 1998).

Even though the secreted *T. reesei* cellulases and hemicellulases are glycoproteins of substantial biotechnological interest, only some degree of information is available on the sites, type, and composition of glycosylation on these enzymes. Glycosylation in *Trichoderma* has been best studied with the main cellobiohydrolase I (CBHI) for which both the N-and O-glycosylation has been characterized. Analysis of the N-linked sugars of CBHI after enzymatic release from the protein has been carried out for CBHI from two high cellulase-producing mutant strains *T. reesei* VTT-D-80133 (Salovuori et al. 1987) and RutC-30 (Maras et al. 1997) both showing high mannose type *N*-glucans. No quantitation of the sugar to protein ratio made in either case. The report by Maras et al. (1997) also revealed an outer branch α-1,6-phospodiester-mannose on the N-linked sugars of CBHI. On the other hand, analysis of CBHI from a genetically modified *T. reesei* strain ALKO2877 (Harrison et al. 1998) indicated very low amounts of modified high mannose N-linked oligosaccharides. Instead, three of the four potential N-glycosylation motifs were occupied by a single *N*-acetyl glucosamine residue. Occurrence of single *N*-acetyl glucosamine residues has also been reported for CBHI purified from the strain *T. reesei* QM9414 (Klarskov et al. 1997).

Mass spectrometry of the CBHI from *T. reesei* strain ALKO2877 revealed heterogeneity in the O-glycosylation of the enzyme linker region and the average number of hexoses attached to the linker peptide was 22 (Harrison et al. 1998). Subsequent monosaccharide analysis showed that the glycosylation of the 8–10 kDa linker peptide was predominantly mannose. Glycosite analysis (sequencing through glycosylated peptides) of the linker revealed that all the threonines on the linker were glycosylated by one to three mannoses. Both serines in the linker were also mannosylated. In addition, linker region of the genetically modified ALKO2877 was sulphated.

Considering the above and that at least the CBHI enzyme analyzed from *T. reesei* ALKO2877 and Rut-C30 was from a culture grown on the same carbon source, lactose, it seems evident that different *Trichoderma* strains glycosylate differently. However, these strains are also high cellulose-producing mutants, which have been developed by the application of several rounds of mutagenesis and screening (reviewed in Nevalainen et al. 1998). Therefore, there is a possibility that they carry yet unknown mutation(s) in their glycosylation machinery. The occurrence of single *N*-acetyl glucosamines in the CBHI of *T. reesei* QM9414 and

ALKO2877 could be explained by a defect in the assembly of *N*-oligosaccharides in the dolichol pathway or by the presence of an endo-β-*N*-acetyl-D-glucosaminidase (EndoH) type trimming activity in *Trichoderma*, which would leave a single *N*-acetyl glucosamine sugar attached to the asparagines (Barreaud et al. 1995). Fascinatingly, such enzyme activity has been found in some filamentous fungi including *Aspergillus oryzae*, *Fusarium oxysporum*, and *Mucor hiemalis* (Suzuki et al. 1995) and appears to be involved in deglycosylation of the *T. reesei* endoglucanase III (EGIII) secreted from an EGIII overproducing*Trichoderma* host (Bower et al. 1998).

In order to successfully produce (near)authentic heterologous proteins in *Trichoderma*, good knowledge of the mechanism of protein glycosylation and nature of the glycans produced by the host is of primary importance. The enzymatic mechanism for protein glycosylation in *T. reesei* has been studied (Maras et al. 1998; van Petegem et al. 2001) and specific genes isolated (Kruszewska et al. 1998; Kruszewska et al. (2000); Maras et al. 2000) with a view of modification of glycosylation to suit for a definite heterologous gene product. Importantly, at least some *T. reesei* strains appear to produce core glycans suitable for extension to glycan structures of mammalian type and the *in vivo* synthesis of complex *N*-glycans with terminal *N*-acetylglucosamine residues has been demonstrated with *T. reesei* Rut-C30 (Maras et al. 1999).

3.4 Expression of Heterologous Thermophilic Enzymes

Enzymes from thermophilic organisms, functional at high temperatures, have characteristics suitable for a number of industrial processes such as paper mills where enzymes active up to 85°C would be desirable for their use in pulp bleaching. Therefore, enzymes from thermophilic microorganisms have been studied and genes encoding e.g., hemicellulases isolated from a number of thermophilic bacteria and some thermophilic fungi. Unfavorable production economics associated with the cultivation of thermophilic microorganisms on a large scale has led to the testing of industrially exploited microorganisms such as *T. reesei* as production hosts for their enzymes.

In general, the best yields for bacterial proteins secreted from fungi have been of the order of 10–20 mg/l (Gwynne et al. 1987). Published reports on the expression of genes encoding thermophilic or thermostable enzymes in *T. reesei* include a xylanase gene from the actinomycete *Actinomadura flexuosa* (Paloheimo et al. 1998), a xylanase gene, *bcx-2,* from *Bacillus circulans* (White and Hinde 1996), modified to increase thermostability of the enzyme (Campbell et al. 1995), the xylanase gene, *xynB,* from *Dictyoglomus thermophilum* (Te'o et al. 2000), and the *xyn2* gene from the thermophilic fungus *Humicola grisea* var. *thermoidea* (Faria et al. 2002). All these genes were expressed under the strong cellobiohydrolase I, *cbh1,* promoter.

In case of the *A. flexuosa* xylanase, the enzyme was produced as a fusion protein to the *T. reesei* mannanase core-linker containing amino acid sequences for KEX2-like processing (Paloheimo et al. 1998). The thermophilic protein was effectively cleaved from the carrier and the reported improvement over 200-fold when compared to xylanase levels in the gene donor. The recombinant xylanase retained its thermal properties. The XYNII enzyme from *H. grisea* was also shown to be correctly processed in the heterologous host *T. reesei* and best yields (about 0.5 g/l) in shake flask cultivations were obtained from a transformant where *xyn2* was fused directly to the CBHI secretion signal (Faria et al. 2002).

Expression of the bacterial *B. circulans bcx-2* and the *D. thermophilum xynB* genes in *T. reesei,* suggest that transcription of the foreign gene may be hampered. For example, there are three codons in the *B. circulans bcx-2* gene, GTA (Val), ATA (Ile), and TTA (Leu) that are never used in the *Trichoderma xyn1*, *xyn 2*, or *cbh1* (reviewed in Bergquist et al. 2002a) and the overall AT-content of *xynB* is 61% compared to less than 40% in a typical *T. reesei* cellulase gene with A or T dominating at the wobble position (Morris et al. 1998). This may cause formation of truncated mRNA transcripts in the fungal host due to incorrect processing of AU-rich elements and possible underpresentation of isoacceptor tRNAs for effective peptide synthesis (Gouka et al. 1996). In *Actinomadura* genes for which a DNA sequence is available, there is a strong bias towards C at the wobble position, which resembles that seen in the powerfully expressed *T. reesei cbh1* (reviewed in Bergquist et al. 2002a).

The codon usage can have a drastic effect on gene expression in *T. reesei*, as demonstrated by Faria et al. (2002) who reconstructed the *D. thermophilum xynB* gene to accommodate the codon usage pattern of *Trichoderma cbh1*. Twenty codons were altered by primer extension-PCR (Te'o et al. 2000). This strategy resulted in effective expression of the *xynB* gene in *T. reesei* and about 12-fold increase in the thermophilic xylanase activity when compared to the untransformed host (Bergquist et al. 2002b).

In summary, the three examples discussed previously, indicate potential bottlenecks in the expression thermophilic bacterial xylanase genes in *Trichoderma*. With the *Actinomadura* xylanase, the carrier protein strategy produced the best results. Expression of the AT rich *D. thermophilum xynB* gene in *T. reesei* required construction of a synthetic gene according to the codon usage of *Trichoderma* genes whereas fusion to a mature endogenous carrier protein did not improve secreted enzyme yields. Finally, production of the *B. circulans* xylanase in *Trichoderma* seemed to suffer from inefficient cleavage of the secretion signal, and possibly, from the presence of four codons not used in effectively expressed *Trichoderma* genes.

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4 HYDROPHOBINS AND THEIR APPLICATION POTENTIAL

4.1 Hydrophobin Properties

Hydrophobins are small amphipathic proteins found uniquely in filamentous fungi (Wösten and Wessels 1997; Wösten 2001). They can be found on fungal spore and cell wall surfaces, where they form layers that may consist of rodletlike structures. They can also be secreted to the culture medium where they assemble on water–air interfaces. Hydrophobins contribute, for instance, to spore rigidity and hydrophobicity, fungal attachment to surfaces, and the growth of fungal aerial hyphae. Hydrophobins contain eight cysteine residues in a characteristic pattern involved in S–S bridge formation. Hydrophobins can be classified into two groups based on the hydropathy profiles deduced from the primary sequences. They have highly interesting physico-chemical properties such as surface activity and self-assembly. Upon assembly on a surface, an amphipathic protein layer is formed that changes the wettability of the solid surface from hydrophobic to hydrophilic and vice versa. For example, the *Schizophyllum commune* SC3 hydrophobin that belongs to class I, can coat Teflon so strongly that it can withstand treatment with the boiling detergent sodium dodecyl sulphate (SDS). Several application possibilities for hydrophobins have been suggested, ranging from protein immobilization and surface modification to their use as emulsifiers in food processing.

The first hydrophobin gene of *T. reesei* was recovered in a search for genes that were highly expressed on glucose medium (Nakari et al. 1993). This *hfb1* gene (Nakari-Setälä et al. 1996) was subsequently used as a probe to isolate the *T. reesei* genes *hfb2* (Nakari-Setälä et al. 1997) and *hfb3* (T. Nakari-Setälä and E. Rintala, personal communication 2001), and the *shr1* gene from a biocontrol strain of *T. harzianum* (Muñoz et al. 1997). These hydrophobin proteins are 7–8 kDa in size with amino acid identity/similarity around 56–65%, and belong to the hydrophobin class II. An interesting hydrophobinlike molecule QIDIII has been characterised from cell walls of *T. harzianum* CECT 2413 that contains only seven of the eight cysteines (Lora et al. 1995).

The hydrophobins HFBII and SHRI can be found on spore surfaces and are expressed in sporulating cultures (Muñoz et al. 1997; Nakari-Setälä et al. 1997). HFBI, as well as HFBII, can be found secreted to the cell walls of vegetative cultures and are also released to the culture medium. The difference between these two is, however, that while HFBI is expressed and produced on glucose containing media, HFBII can be found in larger quantities in cultures grown on lactose or cellulose (Askolin et al. 2001; Nakari-Setälä et al. 1996; Nakari-Setälä et al. 1997). The proteins can be extracted from the cell walls with ethanol or SDS, and interestingly, recovered in the foam after bubbling the culture medium with air (Askolin et al. 2001; Nakari-Setälä et al. 1996; Nakari-Setälä et al. 1997). Reasonable amounts of hydrophobins can be obtained and the amounts can be increased to close to 1 g/l level by expressing the genes using stonger promoters such as the *cbh1* promoter (Askolin et al. 2001; Bailey et al. 2002). Deletion mutants of *hfb1* and/or *hfb2* revealed that the corresponding proteins both contribute to the hydrophobicity of the fungal hyphae, and HFBI to colony morphology and formation of aerial hyphae (S. Askolin, personal communication 2002). Deletion of *hfb2* reduced spore hydrophobicity and sporulation frequency.

It is interesting that HFBI and HFBII, despite both belonging to the hydrophobin class II, show distinct biochemical properties. Neither of them seem to self-assemble to form clear rodletlike structures on spore or hyphal surfaces like SC3 belonging to class I does, but pure HFBI and HFBII can assemble to form fibrillar structures on solid surfaces (Torkkeli et al. 2002). As analysed by small angle x-ray scattering (SAXS), the HFBII crystals are formed of highly ordered structures. Both hydrophobins assemble to tetramers in solution but in dilute solutions HFBII appears preferentially as monomers (Torkkeli et al. 2002). HFBI and HFBII also assemble on solid surfaces to make a uniform protein layer (Linder et al. 2002). The assembly property of HFBI seems stronger than that of HFBII, but layers of both HFBI and HFBII can be visualised with atomic force microscope (AFM) (A. Paananen and M. Linder, personal communication 2002). The self assembly and surface activity of hydrophobins is also demonstrated by their good separation in aqueous two-phase systems (ATPS) to the nonionic surfactant phase, HFBI showing an extreme separation behavior (Linder et al. 2001). Due to their surface activity and aggregation properties hydrophobins are not easy to purify by conventional means and ATPS thus provides an excellent technique for purification of in particular HFBI. Hydrophobins are generally difficult to produce and purify in reasonable amounts for application trials. The good expression levels and purification schemes established for the *T. reesei* hydrophobins HFBI and HFBII now enable these interesting proteins to be studied in detail to examine the basic physico-chemical properties as well as their application potential.

4.2 Hydrophobin Applications in Protein Production and Purification

The extreme separation of hydrophobins in ATPS is so strong that fusion proteins having HFBI as a partner (T. Nakari-Setälä and M. Linder, personal communication 2002; Collén et al. 2002a; Collén et al. 2002b) and even yeast cells expressing HFBI on the cell surface (Nakari-Setälä et al. 2002) separate efficiently in nonionic ATPS systems. Fusion to HFBI thus provides a cheap, easy, and efficient way to purify a protein of interest from the extracellular enzyme mixture produced by *Trichoderma*, and this approach could also be useful for proteins produced in other organisms.

The approach was tested by fusing C-terminally the coding region for HFBI to the complete endoglucanase EGI with

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the CBD, or to the EGI core-linker region. These multidomain molecules were efficiently produced and secreted by *T. reesei* with the *cbh1* promoter at gram/liter level (M. Qiao, personal communication 2000). The whole culture broth was subjected to ATPS and the fusion protein recovered almost exclusively at the top phase while the other extracellular proteins (endogenous EGI included) remained in the bottom phase. It seems that the fusion protein is even more efficiently secreted to the culture medium than HFBI itself, which remains partly in the fungal cell walls, and causes less foaming than HFBI (see later). Nevertheless, the separation properties and the fact that the catalytic activity of the EGI–HFBI fusion towards small soluble substrates remains unaltered indicates that domain folding has occurred correctly. ATPS separation of EGIcore–HFBI fusion produced by *T. reesei* succeeded also from pilot scale bioreactor cultures directly from the whole culture broth including fungal mycelium (Selber et al. 2002), showing the industrial potential of the system. Some industrial applications may not require cleavage of the protein of interest from the hydrophobin part. The fusion can be cleaved by conventional means using e.g., specific proteases, and the two protein components subsequently separated in another round of ATPS leading to the recovery of the product in the water phase.

Being surface active proteins, hydrophobins contribute to foam formation in shaken cultures, e.g., in aerated bioreactors. This leads to demand of the use of antifoam, complicates the process, and may interfere with product formulation. Bailey et al. (2002) studied *T. reesei* strains inactivated in or overexpressing the *hfb1* or *hfb2* genes. Overexpression of the hydrophobins led to increased foaming during the cultures whereas deletion of the *hfb2* gene in particular reduced foaming significantly. Production strains inactivated in extracellular hydrophobin production can have advantages in industrial enzyme production. That hydrophobins are highly surface active and contribute to the formation and stability of foam is also demonstrated by the finding that gushing of beer is caused by fungal (e.g., *Fusarium*) hydrophobin contamination of barley used in wort preparation, and that gushing can also be seen when small amounts of *T. reesei* hydrophobin is added in beer bottles (Kleemola et al. 2001).

4.3 Hydrophobin Immobilization Applications

The assembly properties of hydrophobins would make them suitable for surface modifications and immobilisation purposes. Linder et al. (2002) studied the adhesion behavior of the two *T. reesei* class II hydrophobins HFBI and HFBII, as isolated proteins and as fusion partners with the endoglucanase EGIcore. HFBI caused the fusion protein to efficiently immobilize to hydrophobic surfaces, such as silanized glass and Teflon. The properties of the surface-bound proteins were analyzed by the enzymatic activity, by surface plasmon resonance (Biacore), and by a quartz crystal microbalance. HFBI fusions formed a tightly bound, rigid surface layer on the hydrophobic support. Binding most likely occurred as a monolayer, with calculated amounts of 14 mg/m^3 of EGIcore–HFBI (2.9–4.4 mg/m^3 of the native hydrophobins). The endoglucanase activity was retained upon binding, which indicates that both fusion domains had folded correctly. The work also demonstrates that through hydrophobins enzymes can be immobilised in active form, which is not always the case when direct immobilization of enzymes is attempted. Although isolated HFBII alone bound surfaces efficiently, it failed to mediate immobilization as a fusion partner. This demonstrates the different characteristics of the two hydrophobins. It was also shown that the HFBI domain caused the fusion protein to polymerize in solution, possibly to a decamer structure, whereas HFBII did not support polymerization (Linder et al. 2002).

The strong immobilization properties of HFBI may even be beneficial in immobilization of cells. Nakari-Setälä et al. (2002) expressed HFBI on the yeast surface by fusing it to the flocculation protein FLOI of *S. cerevisiae*, replacing simultaneously the FLOI lectin domain. Although expression and production of the FLOI–HFBI fusion protein reduced yeast growth, the protein appeared to be correctly exposed to the yeast cell surface. The recombinant yeast cells separated in ATPS consisting of a nonionic polyoxyethylene detergent $C_{12\text{-}18}EO_5$ more efficiently than the nontransformed yeast. Determination of cell surface characteristics using contact angle and zeta potential measurements indicated that HFBI producing cells were more apolar and slightly less negatively charged than the parent cells. A two-fold increase in the binding affinity of the hydrophobin producing yeast to hydrophobic silicone-based materials was observed while no improvement in the interaction with hydrophilic carriers could be seen when compared to the parent cells (Nakari-Setälä et al. 2002). It is possible that hydrophobin producing yeast strains could have a benefit in processes where yeast immobilization is desired, by allowing higher biomass and more stable attachment.

5 CONCLUSIONS

The *Trichoderma* community is not very large but the worldwide research efforts have been well focused, concentrating on basic and applied aspects of those features of *Trichoderma* that make it an outstanding industrial organism. The high interest and demand for sustainable development and use of renewable resources such as lignocellulosics as raw material for the production of various biocatalysts and chemicals suit *Trichoderma* very well and new applications and technologies can be built on the very sound knowledge-base we already have on this organism. The more recently discovered proteins, hydrophobins, have exceedingly interesting properties and have been until now only found in filamentous fungi. It remains to be seen whether potential future applications of hydrophobins will come close in importance to those of the fungal enzymes applied so vastly in industry today.

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Several fungal genome sequences are becoming available, and there is also interest to proceed with the sequencing of *T. reesei* and *Trichoderma* species used as biocontrol agents. Currently, EST sequencing of is being carried out by companies exploiting *Trichoderma* and in Brasil (http://trichoderma.iq.usp.br/TrEST.html). The recent announcement by Joint Genomics Institute (USA) indicates that we can expect the complete genome sequence of *T. reesei* during the year 2003 (http://www.jgi.doe.gov/). First transcriptional profiling experiments with the available *T. reesei* EST sequences have already been published (Chambergo et al. 2002), and proteomics methods established (Lim et al. 2001; T. Pakula, personal communication 2001). These developments would provide means to take the *Trichoderma* research to a new level in the future. The genome data will provide entirely new possibilities to understand the versatility and complexity of the enzyme systems and their regulation, as well means to study physiology and the reasons why *Trichoderma* is such an efficient producer of proteins at an industrial scale.

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Plectomycetes: Biotechnological Importance and Systematics

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1 INTRODUCTION

The Ascomycota consisting almost 40% of known fungal species (ca. 80,000) is the largest phylum in the kingdom Fungi (Kirk et al. 2001). Within the Ascomycota the plectomycete species diversity is about 900 species accommodated in 90 genera, (including their related anamorphic species). Plectomycetes are of great importance for the following reasons: (a) they live in soil as saprophiles and decompose various substrates from sugar to cellulose and keratine, (b) useful in fermentation and related industries, (c) some of them cause mycosis or produce mycotoxins, and (d) the trichocomaceous species *Aspergillus nidulans* with an *Emericella* teleomorph is one of the model fungi for genetic studies and related disciplines. This chapter outlines the current status of plectomycete systematics, phylogeny, and evolution with emphasis on the economic importance of *Aspergillus* and *Penicillium*, and their related teleomorphs.

2 BIOTECHNOLOGICAL IMPORTANCE OF THE PLECTOMYCETES

Plectomycetes, particularly members of the Trichocomaceae has well developed enzyme systems that are used in the beverage and food industries. Since the first enzyme takadiastase (invented by Jokichi Takamine) by *A. oryzae* was industrially produced in 1894, glucoamylase and amylase from black *Aspergillus* spp., pectinase from *A. niger*, and *Penicillium notatum* and proteases by *Aspergillus* spp. have been used in food processing (Berka et al. 1992; Novo-Nordisk Co.—http://www.novo.dk/enzymes/Technology.htm). Beneficial members of *Aspergillus* are also important organisms in Japanese alcoholic beverage and fermentation industries such as sake (*A. oryzae*), shochu (Japanese sweet potato's and bareley's spirits; *A. saitoi*, *A. kawachii*), awamori (Okinawa's rice shochu; *A. awamori*, *A. saitoi*), shoyu (soy sauce: *A. oryzae*, *A. tamarii*), miso (*A. oryzae*), and katsuobushi (dried bonito: *A. glaucus*) (Hara et al. 1992). In addition to these, *A. terreus* is industrially used for itaconic acid, and *A. niger* for citric acid and gluconic acid productions (Roehr et al. 1992).

On the other hand, some species of *Penicillium* are used as processing agents primarily in the cheese and sausage-making. A white mold *P. camemberti* is used for the Camembert type cheese making, whereas a greenish blue mold *P. roqueforti* is used for the Roquefort type cheese. The pharmaceutical industry of penicillin produced by *P. chrysogenum* and its semisynthetic derivatives continues to be important fungal-produced antibiotics. *P. griseofulvum* is used for the commercial production of griseofulvin, oral and topical antibiotic, which is effective against dermatophyte infections. Recently, pravastatin (the commercial name "mevalotin") production with the use of *P. citrinum* as the first step and *Streptomyces carbophilus* as the second step in fermentation is worthy of special mention (Tsujita et al. 1997). Pravastatin significantly reduces plasma cholesterol levels in a variety of mammals, including man and effective in the therapy of hypercholesterolemia.

The trichocomaceous species *Monascus purpureus* (=*M. ruber*) is important in the production of Asian fermented foods, e.g., red rice (angkak), soya bean cheese (Orient cheese), food-coloring pigments (Hawksworth and Pitt 1983), and antihypercholesterolemic agent, (Endo 1991). Monacolin and lovastatin (the commercial name "Mevacor") produced from a *Monascus* are assignable to the same

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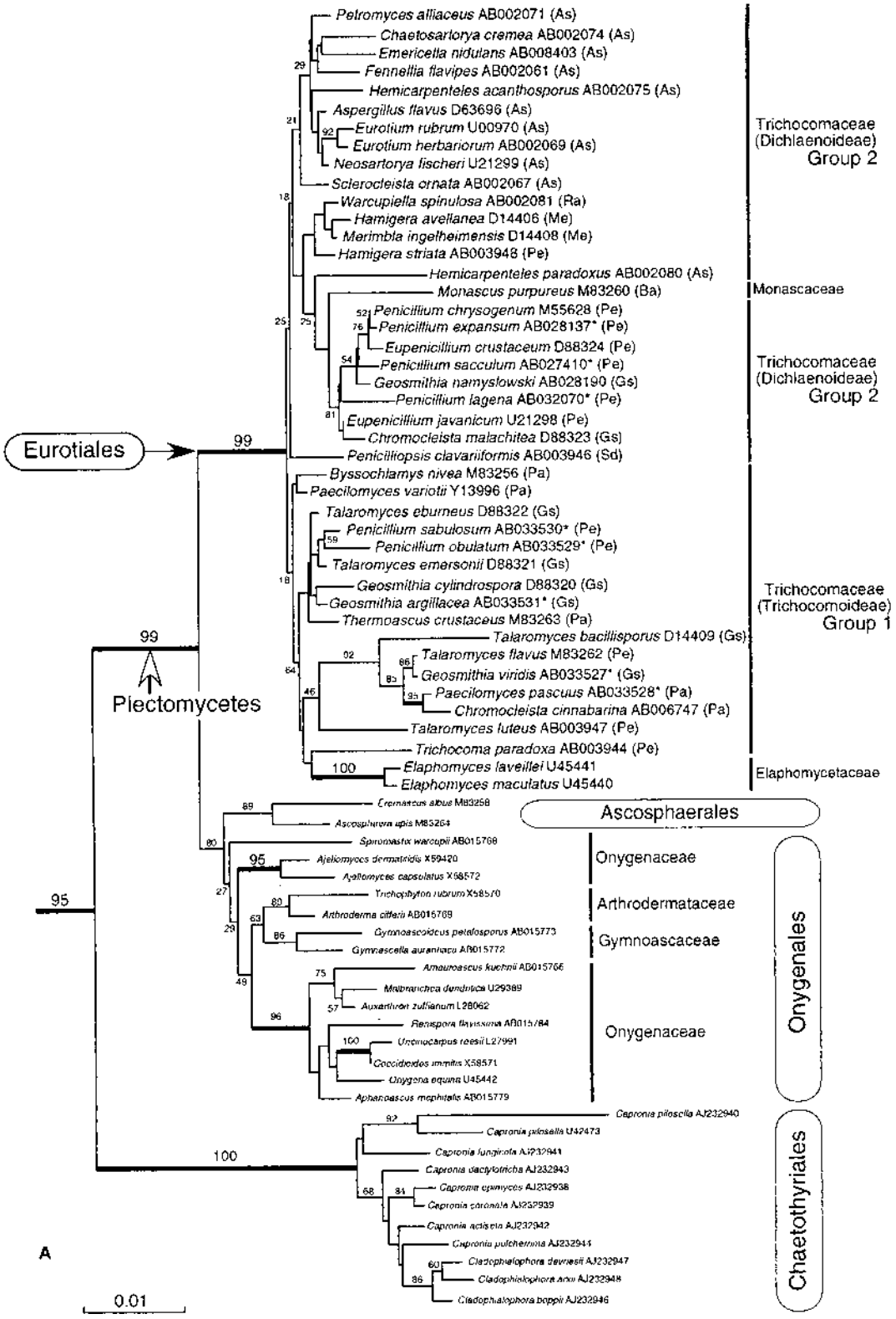

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compound (U.S. Food and Drug Administration public docket, No. 97P-0441 in 1998).

Several *Aspergillus*, *Penicillium*, *Paecilomyces*, and relatives cause biodeterioration of foods, fruits, blubs, textiles, and cultural properties such as painting and old books (Arai 1990; Domsch et al. 1980; Pitt and Hocking 1997). Xerophilic species *Eurotium* and its *Aspergillus* anamorphs damage lens and swords (Otsuki 1962). Several members of *Aspergillus*, *Penicillium*, and relatives are the causal organisms of "opportunistic infections" in humans and animals (e.g., aspergillosis by *A. fumigatus* and *A. flavus*, penicilliosis by *P. marneffei*) (Howard and Miller 1996). Mycotoxins such as aflatoxins (*A. flavus*, *A. parasiticus*), citrinin (*P. citrinum*), and patulin (*P. patulinum*) are important as food-safety agents (Howard and Miller l.c.).

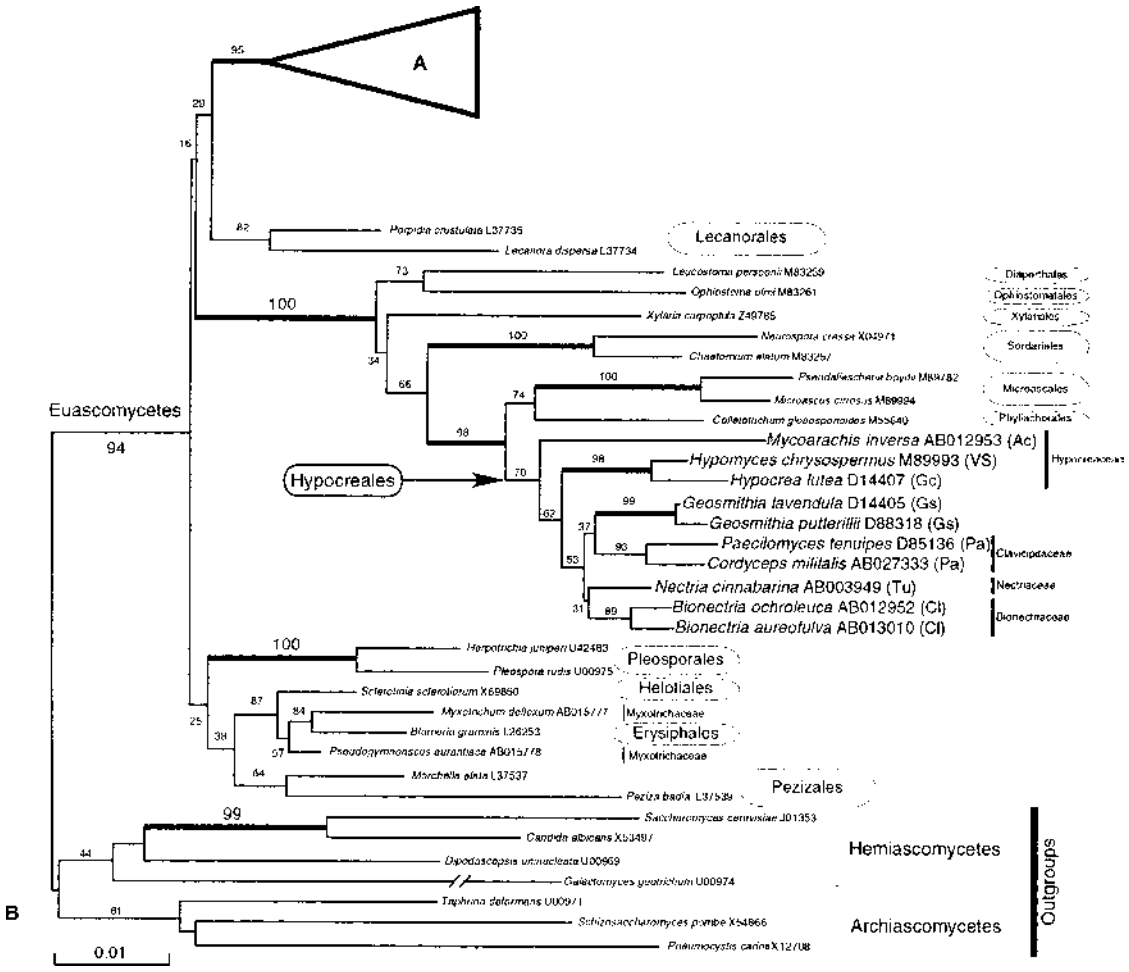

Figure 1 Phylogenetic relationships of 99 euascomycetous species with seven archiascomycetous and hemiascomycetous species as outgroups. Well-aligned 1378 sites of SSU rDNA sequences were used for this analysis. The tree was constructed using the neighbor-joining method (Saitou and Nei 1987) based on the distances calculated by the Kimura's two parameter model (Kimura 1980) with transition/transversion ratio = 2.0. Bootstrap (Felsenstein 1985) percentage derived from 1000 replicates are indicated for the selected nodes. The alignment, tree construction, and bootstrapping were carried out on the Apple PowerMac computer equipped with G4 processor, using Clustal X (Thompson et al. 1997). The scale bars indicate one base change per 100 nucleotide positions. Asterisks after DNA data bank accession numbers indicate the new sequences determined by Ogawa et al. (unpublished). Other rDNA sequences were retrieved from the DNA data bank; the respective accession numbers are indicated after the Latin name. (A) The portion of the sister clades the monophyletic Plectomycetes and Chaetothyriales. (B) The basal portion of the whole tree including selected species of the Hypocreales and other orders in the Ascomycota and seven outgroup taxa from the archiascomycetes and hemiascomycetes.

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3 ADVANCES IN PLECTOMYCETE SYSTEMATICS

Very recently, Geiser and LoBuglio (2001) have provided an overview of plectomycete systematics and related topics. Therefore, we will very briefly trace the advances of plectomycete systematics based on different taxonomic approaches.

3.1 Morphology-Based Systematics

Traditionally plectomycete taxonomy and classification have been performed using morphological characters (e.g., Mayden 1997). The biological species concept that defines species as groups of interbreeding individuals using tester strains is powerless for many plectomycetes, except for part of gymnoascalean fungi (e.g., *Arthroderma* = *Nannizzia*). The morphology-based class Plectomycetes in the subphylum Ascomycotina (Fennell 1973) was defined only on the basis of the cleistothecium and gymnothecium (rarely naked asci as seen in *Byssochlamys*), and related anamorphs (=mitotic states) and was composed of nine families, i.e., Amorphothecaceae, Gymnoascaceae, Onygenaceae, Monascaceae, Thermoascaceae, Trichocomaceae, Eurotiaceae, Cephalothecaceae, and Pseudeurotiaceae in the single order Eurotiales. The concept of class was overturned by Malloch (1981) and Malloch and Cain (1972; 1973) with emphasis on structure and evolutionary simplifications and modifications. Malloch (1981) included the Eurotiaceae in the Pleosporales, the Pseudeurotiaceae in the Pleosporales, the Trichocomaceae in the Hypocreales, and both Monascaceae and Onygenaceae in the Pezizales. On the other hand, Benny and Kimbrough (1980) recognized six orders: Ascosphaerales, Elaphomycetales, Eurotiales, Microascales, Onygenales, and Ophiostomatales, including 12 families developing a plectomycete centrum. Subsequently no categories for the above orders for all ascomycetes have been used to emphasize circumscription of monophyletic taxa at the ordinal level. This practice has been adopted within the last several years (e.g., Hawksworth et al. 1995; Alexopolous et al. 1996). Although morphological characters are basically important in fungal systematics, however, the resolution by the traditional morphological approaches to systematics in plectomycetes and related anamorphs was limited, and a need for newer approaches had become apparent to construct the taxonomic system reflecting phylogeny and evolution. Several examples are shown in the following sections.

3.2 MOLECULAR SYSTEMATIC AND PHYLOGENETIC APPROACHES

3.2.1 Molecular Taxonomy and Chemotaxonomy

Sexual and asexual reproductive structures have provided important phenotypic characters to measure relatedness and evolutionary affinities among fungi. If the fungi lose reproductive structures, accurate taxonomic assignment is quite difficult even at the phylum level. The roots of the current fungal systematics and phylogenetics are molecular taxonomy (Kurtzman and Phaff 1987) and chemotaxonomy (Frisvad et al. 1998; Sugiyama et al. 1991). Initially nuclear DNA base composition (mol% G + C), nuclear DNA relatedness (DNA–DNA hybridization), and electrophoretic comparison of enzymes, and the major component of ubiquinones (Q), which are important carriers in the electron transport chain of respiratory systems have been used to define particularly the species of *Aspergillus*, *Penicillium*, and related teleomorphs (e.g., Kuraishi et al. 1985; 1990; 1991; Kurtzman et al. 1986; Paterson 1998; Sugiyama et al. 1991; Tamura et al. 1998; 1999; Yamatoya et al. 1990). However, these characters resolve only to the species level and are not useful in grouping genera in higher taxa.

Kimura's neutral theory of molecular evolution provided an impact on phylogeny and evolution of organisms (Kimura 1983). In the 1980s, development of molecular biological techniques (particularly gene cloning, nucleic acid sequencing, and polymerase chain reaction), proliferation of high performance computers, and improvement of molecular evolutionary analysis programs have extended studies on relatedness, phylogeny, and evolution of fungi at the molecular (or gene) level (see Sugiyama 1998). In the early 1990s, fungal molecular taxonomy and chemotaxonomy steered toward fungal molecular systematics (Bruns et al. 1991; Hillis et al. 1996; Reynolds and Taylor 1993) and further fungal molecular phylogenetics (Berbee and Taylor 1999; 2001) added a new dimension to the understanding of relationships between the different ascomycete orders and higher taxonomic categories.

Ribosomal RNA (rRNA) and its template ribosomal DNA (rDNA) sequence comparisons, as a nucleic acid character and "evolutionary clock," offer a means for estimating both close and distant relationships among lower to higher fungi (e.g., Berbee and Taylor 1999; 2001; Bruns et al. 1991; Sugiyama 1998). Phylogenetic analysis among distantly related taxa, i.e., at the class–phylum–kingdom level, using small subunit rRNA gene (SSU rDNA or 18S rDNA) sequence divergence has contributed to well-resolved and statistically supported conclusions (see Berbee and Taylor 1999; Sugiyama 1998). Historically molecular phylogenetic studies of plectomyceteous fungi using rRNA sequencing dated back to the beginning of the 1990s. Chang et al. (1991), Dupont et al. (1990), Logrieco et al. (1990), and Peterson (1993) independently reported data on rRNA partial sequence comparisons for selected species of *Aspergillus*, *Penicillium*, and related teleomorphs. Phylogenetic analyses among distantly related fungal taxa at the class–phylum–kingdom level using SSU rDNA shifted to the full sequences from partial ones. Based on SSU rDNA full sequences, Berbee and Taylor (1992b) demonstrated that representative taxa of the cleistothecial and perithecial ascomycetes form the respective monophyletic clades, which have been named the Plectomycetes and Pyrenomycetes, respectively. Furthermore,

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Berbee and Taylor (1993) estimated the absolute time of the origin of major groups of fungi from the SSU rDNA sequence divergence as a molecular clock and fossil records (see Section 7.2). Subsequent advances for investigating relationships among higher taxa of Plectomycetes such as orders and families have been mentioned in the following sections.

The species concepts in Plectomycetes are old-fashioned but now gaining importance because of the inclusion of economically and medically important taxa. Initially the major target was *A. flavus* and closely related species in section *Flavi* in the relation to the aflatoxin problems (Hicks et al. 2002; Howard and Miller 1996). Klich and Pitt (1988) suggested that these *Aspergillus* species can be phenotypically distinguished using the phenotypic (morphological and biochemical) species concept. Such an approach is useful in the identification, but it can measure the genetic distance between species. Strong concordances between independent molecular systematic data from DNA relatedness (Kurtzman et al. 1986; Tamura et al. 1998), electrophoretic comparison of enzymes (Yamatoya et al. 1990), and DNA sequences of protein-coding genes (Geiser et al. 1998), ITS region (Nikkuni et al. 1998), and the major ubiquinone system as a chemotaxonomic character (Kuraishi et al. 1990; Rigo et al. 2002) have been demonstrated. In conclusion, *A. oryzae* and *A. sojae* appear to be domesticated nonaflatoxigenic strains of *A. flavus* and *A. parasiticus*, respectively (see later). Therefore, Saito's hypothesis (Saito 1943) on the relationship between the wild mold *A. flavus* and the koji mold *A. oryzae* is supported by modern molecular systematics as pointed out by O'Donnell (1999) and also by molecular population genetic approaches (cf. Section 7.1 in this chapter).

4 PLECTOMYCETES AND THEIR ORDERS

4.1 Detection of a Monophyletic Plectomycetes

Phylogenetic trees inferred from SSU rDNA sequence divergence indicate the existence of three major lineages within the phylum Ascomycota (see Sugiyama 1998). Here, we will demonstrate our newest SSU rDNA sequence-based phylogeny as inferred from 107 taxon samples in the center of plectomycetous species, including seven archiascomycete and hemiascomycete species as groups, within the Ascomycota (Figure 1A,B). Although we will discuss more for the respective taxa of Figure 1 in the following sections, this tree's topologies, supporting to the monophyly of the Plectomycetes (99% bootstrap confidence) and the basal position of *Capronia–Cladophialophora* in the Chaetothyriales (see Geiser and LoBuglio 2001) are almost the same as those of Geiser and LoBuglio (l.c.), Ogawa et al. (1997), and Sugiyama (1998). In all trees the plectomycetes and pyrenomycetes formed the respective monophyletic groups that were well supported by bootstrapping (95% or greater). Geiser and LoBuglio (l.c.) adopted the term "the monophyletic Plectomycetes" as previously suggested by Berbee and Taylor (1992b; 1994). In this chapter, we will follow Geiser and LoBuglio's treatment. Our discussions on higher ranking, above the class level, will be made elsewhere in the near future.

4.2 Plectomycete Orders

Recent phylogenetic analyses of SSU rDNA sequences has indicated that the Plectomycetes sensu Geiser and LoBuglio (2001) and Eurotiomycetes sensu (Eriksson 2001; any tree was not shown) contain three orders, i.e., Ascosphaerales, Onygenales, and Eurotiales. Elaphomycetales was accommodated within the Eurotiales as the family Elaphomycetaceae. At the moment, we follow Geiser and LoBuglio's (l.c.) and Eriksson's (l.c.) systematic placement on Elaphomyces. Systematics profiles of the respective orders are briefly given below.

4.2.1 Ascosphaerales

Members of this order are characterized by acellular ascomata and are yeast-like. The order contains two families Ascosphareraceae and Eremascaceae, typified by *Ascosphaera* and *Eremascus*, respectively. The former family is separated from the latter one by unique spore balls in a transparent spore cyst or nutriocyte. Recent SSU rDNA sequences analyses (Geiser and LoBuglio 2001; Sugiyama 1998; Figure 1) show that *Ascosphaera* and *Eremascus* form a monophyletic group with comparatively high statistical support, which is far from the monophyletic Eurotiales. Kirk et al. (2001) treated both Ascosphaeraceae and Eremascaceae as Incertae sedis within the Eurotiales, whereas Eriksson (2001) accommodated Ascosphaeraceae and Eremascaceae within the Onygenales. The accurate positioning of Ascosphaerales and the families included is still controversial.

4.2.2 Onygenales

Members of this order are characterized by gymnothecial ascomata with or without unique appendages; anamorphs are arthric; they are ketatinophilic or cellulolytic, some parasitic on humans and other animals, and also in soil. Currah (1985) included the familes Arthrodemataceae, Gymoascaceae, Myxotrichaceae, and Onygenaceae. Among these families, all species of the Myxotrichaceae are saprobic on cellulosic substrates. Subsequently, the Onygenales was restricted to keratinolytic general with distinctive anamorphs (Currah 1994). On the other hand, Udagawa (1997) modified Currah's system (1985) with the ubiquinone system as a chemotaxonomic character and added the Amauroascaceae in the Onygenales. Geiser and LoBuglio (2001) recognized the four families in the Onygenales sensu Currah (1985); however, they mentioned that the branch leading to the Onygenales, suggesting monophyly with weak bootstrap support (less than 50%).

Recent NJ and MP analyses of LSU rDNA partial sequences (ca. 570 bp) suggested that the tree topology was

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concordant with the NJ trees based on SSU rDNA sequences (Sugiyama and Mikawa 2001; Sugiyama et al. 1999b) and corresponded to the classification system of the Onygenales by Currah (1985) and its minor modification by Udagawa (1997), except for the Onygenaceae. Our NJ tree (Figure 1A) indicates that the Onygenaceae is polyphyletic and two representatives the Myxotrichaceae, *Myxotrichum deflexum* and *Pseudogymnoascus aurantiaca*, grouped with the erysiphalean species *Blumeria graminis* and the helotialean species *Sclerotinia sclerotiorum* with comparatively high bootstrap support (87%) (Figure 1B) as already detected by Sugiyama et al. (1999b). As shown in Figure 1B, this family has a discomycete affinity. Eriksson (2001) and Kirk et al. (2001) placed the Myxotrichaceae sensu Currah (1985), accommodating *Byssoascus*, *Myxotrichum*, *Pseudogymnoascus*, and their associated anamorphs as ascomycetes incertae sedis. More taxon samples from the Onygenaceae are needed to elucidate phylogenetic relationships among the onygenalean taxa.

4.2.3 Eurotiales

Eurotiales sensu Fennell (1973) contained nine families, but Malloch (1981) did not recognize the Eurotiales and included the Trichocomaceae and the pyrenomycete family Hypocreaceae in the order Hypocreales (see Section 1). Benny and Kimbrough (1980) recognized the Eurotiales in which the families Trichocomaceae and Cephalothecaceae were included. The Eurotiales sensu Hawksworth et al. (1995) contained five families, i.e., Ascoshaperaceae, Cephalothecaceae, Monascaceae, Pseudeuortiaceae, and Trichocomaceae. Molecular phylogenetics has indicated the circumscription of this order. The former two families Ascosphaeraceae and Eremascaceae have been moved to the Ascosphaerales (see previously), whereas the Pseudeurotiaceae sensu Malloch and Cain (1972), which is more or less equivalent to the Cephalothecaceae sensu Benny and Kimbrough (1980), with thick-walled cleistothecia and *Chalara*-like anamorphs was excluded from the plectomycete lineage (Rehner and Samuels 1994; Suh and Blackwell 1999).

Members of the Elaphomyceteaceae typified with the genus *Elaphomyces* are unique because of hypogeous, mycorrihizal habit, and ascoma size and shape (Benny and Kimbrough 1980). Although these fungi were regarded as plectomycetes or discomycetes (see Geiser and LoBuglio 2001) recent molecular phylogenies (Geiser and LoBuglio l.c.; Ogawa and Sugiyama 2000; Sugiyama 1998) also demonstrate the monophyly of members of the families Elaphomycetaceae (or Elaphomycetales) and Trichocomaceae (=Eurotiaceae) plus Monascaeae, as well supported bootstrap test (99% in Figure 1A). These three families are regarded to be of a plectomycete origin. However, the closest relatives and the phylogenetic position of *Elaphomyces* still remain uncertain.

5 PHYLOGENETIC RELATIONSHIPS AMONG TRICHOCOMACEOUS FUNGI

The Trichocomaceae includes cleistothecial and gymnothecial teleomorphic genera which are characterized by brightly colored asomata, oblate (bivalved) to elliptical ascospores, and produce prominent phialidic anamorphs (Malloch and Cain 1972; Pitt 1995; Pitt et al. 2000). As stated previously, the anamorphs are assignable to *Aspergillus*, *Penicillium*, and relatives of industrial, biotechnological, and medicinal importance; they are saprobic mainly in soil and decay plant materials (Domsch et al. 1980; Udagawa 1987). On the other hand, the Monascaceae represented by *Monascus* is characterized by small, cleistothecial, thin-walled, and a distict peridium composed of flattened cells; the *Basipetospora* anamorphs are unique in conidium ontogeny (Cole and Samson 1979); they are saprobic and prefer osmophilic substrates.

SSU rDNA sequence-based phylogenies (Figure 1A) have strongly suggested for almost all the members of Monascaceae which are included within the Trichocomaceae (cf. Berbee et al. 1995; Sugiyama 1998; Sugiyama et al. 1999b), except for the strictly anamorphic species such as *Basipetospora chlamydospora* of a pyrenomycete origin (Sugiyama et al. 1999b; unpublished). This result suggests that a peculier conidium ontogeny (i.e., holoblastic, retrogressive conidiogenesis; cf. Cole and Samson 1979) seen in Basipetospora was derived from the phialidic one (cf. Berbee et al. 1995). Taxonomically, therefore, two families should also be united into one family (Sugiyama, unpublished).

6 *ASPERGILLUS*, *PENICILLIUM*, *PAECILOMYCES* AND RELATIVES, AND THEIR TELEOMORPHS

6.1 Conidiogenous Structures and Anamorph–Teleomorph Connections

Aspergillus and *Penicillium* characterized by "aspergillum" and "penicillus" as the conidiogenous structure, respectively, are of considerable interest because of the diversity of cleistothecial ascomata and ascospore types in their related teleomorphs (Berbee et al. 1995; Malloch and Cain 1972). The teleomorphs associated with *Aspergillus* anamorphs are assignable to nine genera represented by *Eurotium*, *Emericella*, and *Neosartorya*, whereas those associated with *Penicillium* anamorphs and relatives are assignable to five genera represented by *Eupenicillium* and *Talaromyces* (Pitt et al. 2000; Yaguchi et al. 1993). *Paecilomyces* is morphologically similar to *Penicillium*, but the former is different from the latter by the phialides with a neck-like elongation, the irregular or verticillate branching of the conidiophores and the lack of true green conidia and associated teleomorphs (Samson 1974). The teleomorph genera with *Paecilomyces* anamorphs are *Byssochlamys*,

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Talaromyces, and *Thermoascus* (Pitt et al. l.c.). The existence of interfaces (e.g., *Geosmithia*, *Raperia*) causes taxonomic confusions. The integrated analysis of genotypic (molecular) and phenotypic characters is very useful in order to elucidate their identity and "true" evolutionary relationships. Several paradigm examples are provided in the following sections.

6.2 Is *Aspergillus* Really Monophyletic?

Using 18S, 5.8S, and internal transcribed spacer (ITS) rDNA sequence data, Berbee et al. (1995) and Chang et al. (1991) evaluated that whether *Penicillium* is monophyletic or not. They suggested that *Aspergillus* may be monophyletic, nested among species with *Paecilomyces* and *Penicillium*. Unfortunately, their phylogenetic analysis included only three taxon samples from *Aspergillus* and associated teleomorphs (i.e., *Neosartorya fischeri*, *A. fumigatus*, and *Eurotium rubrum*).

The bootstrapped parsimony tree estimated from SSU rDNA sequence data (1663 bp) by Tamura et al. (2000) suggested that almost all the members of *Aspergillus* and associated teleomorphs are basically monophyletic except for the respective type species *Hemicarpenteles paradoxus* and *Warcupiella spinulosa*, both in section *Ornati*, although the bootstrap confidence is comparatively low (56%). Sugiyama and Ogawa's NJ tree (current chapter) supports the topologies demonstrated by Tamura et al. (l.c.) although the statistical support is lower (21% in Figure 1A).

Exclusion of *H. paradoxus* from the *Aspergillus* lineage is consistent with the ascoma morphology (Pitt 1995; Tamura et al. l.c.) and the major ubiquinone system (Kuraishi et al. 1990). Another species of *Hemicarpenteles*, *H. acanthosporus* (section *Ornati*) and *A. clavatus* (section *Clavati*), both having Q-10 as the major ubiquinone, formed a well-supported clade (98% bootstrap support in Tamura et al. l.c.). The same conclusion was also evidenced in the LSU rDNA D1/D2 region sequence data of Peterson (2000b). Very recently Udagawa and Uchiyama (2002) proposed a new genus *Neocarpenteles* to accommodate *H. acanthosporus* in the light of these molecular and chemotaxonomic data.

On the other hand, *W. spinulosa* which is characterized by gymnothecial (hyphal) ascomata lacking stroma, grouped with the *Merimbla*-producing *Talaromyces avellaneus* (≡*Hamigera avellanea*) with 75% bootstrap support (Tamura et al. l.c.; cf. Figure 1A). This means that the placement of *Warcupiella* in section *Ornati* is questionable. *Raperia*, an interface anamorph genus (Pitt and Hocking 1985) accommodates an anamorph species *A. warcupii* Samson & Gams (≡*A. spinulosus* Warcup) associated with a teleomorph *W. spinulosa*. Their molecular phylogeny suggests a revival of *Raperia* (Pitt et al. 2000) which is distantly related to the majority of *Aspergillus* species.

Phylogenetic and geneological relationships among six subgenera and 18 sections of *Aspergillus* (Gams et al. 1985) have been demonstrated by Peterson (2000b) using LSU rDNA (D1/D2 region) and Tamura et al. (l.c.) using rDNA sequence data, respectively.

6.3 Is *Penicillium* Really Polyphyletic?

As stated above, Berbee et al. (1995) have detected that three representative species of *Penicillium* with a *Talaromyces* or *Eupenicillium* teleomorph are not monophyletic within the Trichocomaceae from 18S, 5.8S, and ITS rDNA sequence analyses. Their phylogeny was consistent with that based on reexamination of 18S rRNA partial sequences (558 bp) by Chang et al. (1991). Subsequently, Ogawa et al. (1997) focused on molecular phylogeny of seven species of the anamorph genus *Geosmithia* with the type species *Geosmithia lavendula* which has been segregated by Pitt (1979a,b) from *Penicillium*. *Geosmithia* includes species lacking a teleomorph as well as species associated with the teleomorphs *Talaromyces* and *Chromocleista* (Yaguchi et al. 1993). Their SSU rDNA sequence (1586 bp)-based NJ analysis (Ogawa et al. l.c.) from 57 selected taxa within the Ascomycota detected that species of *Geosmithia* are polyphyletic with evolutionary affinities to at least three groups of the euascomycetes: i.e., strictly anamorphs *G. lavendula*, the type species of the genus and *G. putterillii* are of pyrenomycetous origin (presumably a hypocrealean origin), whereas other *Geosmithia* species with or without a teleomorph are in the *Talaromyces* and *Eupenicillium* groups in the Trichocomaceae of the Plectomyetes. Both NJ and MP analyses of 5S rDNA sequences (70 bp) also supported the results from the 18S rDNA sequence analyses. In addition, 28S rDNA partial sequence (580 bp)-based NJ and MP analyses showed that, within the Hypocreales, *G. lavendula* and *G. putterillii* grouped with the hypocrealean fungi, cleistothecial, *Acremonium*-producing *Mycoarachis* inversa and *Emericellopsis terricola*, and the strictly anamorph species of *Acremonium*. In their paper, phylogenetic considerations have also been presented for some genera characterized by the plectomycete centrum (Berbee and Taylor 1992a; Ogawa et al. l.c.; Rehner and Samuels 1994; 1995; Spatafora and Blackwell 1993).

Recently, Ogawa and Sugiyama (2000) (Figure 1) have further elucidated phylogenetic relationships of the additional taxon samples of phylogenetically importance in the Trichocomaceae. As a result, the type genus *Trichocoma* is placed at a position relatively close to *Talaromyces*, including the *Penicillium*-producing *Talaromyces luteus*. However, *T. luteus*, showing similar ascospore and anamorph morphology as *Trichocoma paradoxa* (Kominami et al. 1952) is not involved in the *Trichocoma* branch. Consequently *T. luteus* is not the closest relative of *Trichocoma*. The *Sarophorum* producing *Penicilliopsis clavariiformis* is rather close to the *Eupenicillium* cluster and *Aspergillus* producing teleomorphs. *Hamigera* with a *Merimbla* and a *Penicillium* anamorph is closely related to *Eupenicillium* and the *Aspergillus* producing teleomorphs rather than *Talaromyces*. The type species *Chromocleista malachitea* with a *Geosmithia* anamorph (Yaguchi et al. 1993) is placed within the *Eupenicillium* cluster. In contrast, the second species *C. cinnabarina* with a *Paecilomyces* anamorph and two

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species of *Talaromyces* grouped together with 100% bootstrap confidence (cf. 85% in Figure 1A). Molecular phylogenies suggest that *Chromocleista* should be taxonomically reexamined.

Similarly, Peterson's (2000a) performed parsimony analysis on the basis of LSU rDNA (D1/D2 region) sequences showed that "many subgeneric taxa in *Penicillium* are polyphyletic, and that the emphasis placed on penicillus structure in *Penicillium* taxonomy led to polyphyletic classifications." Very recently, Iwamoto et al. (2002) detected the phylogenetic position of the strictly anamorph genus *Thysanophora* using SSU and 28S rDNA partial (D1/D2 region) sequence divergence. Their molecular phylogenies suggest that *Thysanophora* has an affinity with *Penicillium* and *Eupenicillium*, i.e., both anamorph genera are congeneric but they are clearly distinct in morphology.

6.4 Is *Paecilomyces* Monophyletic?

Sugiyama et al. (1999a,b) further looked at whether species of *Paecilomyces* are monophyletic. The heterogeneity of the genus had been shown by Samson (1974). The phylogeny as inferred from SSU rDNA sequences (1576 bp) divided l35 selected taxa into two groups (Sugiyama et al. l.c). The grouping by Sugiyama et al. (l.c.) is corresponding to that in Figure 1A. Group 1 contains teleomorph genera *Byssochalamys*, *Talaromyces*, *Thermoascus*, *Trichocoma*, and *Sagenoma*, whereas group 2 is better supported (>ca 80% bootstrap; but lower confidence 25% in Figure 1A) and contains teleomorph genera *Chaetosartorya*, *Eupenicillium*, *Eurotium*, *Fennellia*, *Hamigera*, *Hemicarpenteles*, *Monascus*, *Neosartorya*, *Penicilliopsis*, *Warcupiella*, and *Xeromyces*. As stated previously, species of *Chromocleista* are accommodated in group 1 or 2. *Paecilomyces* producing *Byssochlamys*, *Penicillium* or *Geosmithia* producing *Talaromyces* and *Thermoascus* spp. are accommodated in group 1, whereas the strictly anamorphic species of *Paecilomyces* in section *Isarioidea* defined by Samson (1974) are placed in the pyrenomycete order Hypocreales (Figure 1B). *Sagenoma* and almost all *Sagenomella* species, including the type species *S. diversispora*, grouped in the *Talaromyces* cluster (group 1), but *S. oligospora* was involved in the Pyrenomycetes. Molecular phylogenetic analyses by Sugiyama et al. (l.c.) have also suggested a polyphyletic nature of the anamorph genera *Paecilomyces* (Samson l.c.) and *Sagenomella* (Gams 1978) as presently delimited.

In conclusion, at the moment, groups 1 and 2 defined by Sugiyama et al. (l.c.) and the authors (current chapter; c.f. Figure 1A) correspond roughly to Malloch's (1985a,b) subfamilies Trichocomoideae and Dichlaenoidea emphasizing the ascospore types (prolate vs. oblate, bivalved) (cf. Berbee et al. 1995). Their ascospore types reflect the evolutionary history of the trichocomaceous taxa. As stated previously, molecular phylogenies until now have suggested that species of the anamorph genera *Aspergillus*, *Penicillium*, *Geosmithia*, and *Paecilomyces* have evolved multiple times from teleomorphs, as opposed to evolving independently (Geiseir and LoBuglio 2001). Only morphology-based taxonomy for strictly anamorph taxa is limited and occasionally makes problematic. Therefore, molecular phylogenetic approaches are essential to elucidate their identity.

7 CURRENT TRENDS AND FUTURE PERSPECTIVES IN PLECTOMYCETE SYSTEMATICS AND EVOLUTION

7.1 Molecular Population Genetic Approaches and Detection of Cryptic Species

In the latter half of the 1990s some researchers in fungal molecular systematics and phylogenetics shifted to analysis of the populations to species level in relation to the species concepts problems, detection of cryptic species, speciation, and internal structure of the species of fungi using strain typing (Taylor et al. 1999a,b). The targets so far are medically important fungi. These are *Coccidioides immitis*, *Histoplasma capsulatum*, *Candida albicans*, *Cryptococcus neoformans*, *A. fumigatus*, and *A. flavus*.

In this chapter, we will focus on the detection of cryptic species in *A. flavus* and closely related species in *Aspergillus* section *Flavi*, which their teleomorphs are still unknown. Geiser et al. (1998) tested the assumptions of clonality and conspecificity in a sample of 31 Australian agricultural soil isolates by assaying restriction site polymorphisms from 11 protein encoding genes (*O*-methyltransferase, β-tublin, calmodulin, and others) and DNA sequences from five of those genes. As a result, (a) *A. flavus*, a good morphological species (Klich and Pitt 1988) fell into two reproductively isolated clades (groups I and II), (b) the lack of concordance among gene genealogies among isolates in one of the clades (group I) was consistent with a history of recombination, (c) five strains (including the ex-neotype isolate) of the closely related industrial fungus *A. oryzae* were clonally related group I (Geiser et al. l.c.). Geiser et al. (l.c.) concluded that nonaflatoxigenic *A. oryzae* isolates is a part of a monophyleitc *A. flavus* clade, and its phenotypic distinctiveness is the result of strong selection associated with domestication. Recently, Geiser et al. (2000) also tested whether two of the variable phenotypic characters known to exist in *A. flavus*, toxin and sclerotium production, correlated with known phylogenetic groupings. These results suggest the limitation of the morphological species concepts and the availability of phylogenetic species concepts. The species concepts problems in ascomycetous fungi have been discussed in the *Gibberella fujikuroi* complex (O'Donnell 1999).

7.2 Molecular Clocks and the Time of Plectomycete Divergences

Finally estimation of the geologic time of the origin of *Aspergillus* and *Penicillium* species is briefly mentioned.

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According to Berbee and Taylor (1993; 2001) the latest calibration using a molecular clock (18S rDNA sequence data) and evidence of fossil records, the ascomycetes and basidiomyces diverged from one another in Paleozoic at about 500 million years ago (mya), 100 mya earlier than the appearance of a pyrenomycete as fossil discovered by Taylor et al. (1999c). The three major lineages (archiascomycetes, hemiascomycetes, and euascomyces) in the Ascomycota established perhaps during the Paleozoic, from 350 to 450 mya. The plectomycetes arose about 250–300 mya in the Permian or Carbobiferous period of the Mesozoic. Diversification in the Trichocomaceae, including economically important species of *Aspergillus* and *Penicillium* is less than 100 mya in the Cretaceous period of the Cenozoic. Very recently, the molecular clock (sequence data for 119 proteins) of Heckman et al. (2001) has indicated that the Pyrenomycetes–Plectomycetes divergence (670 mya) is very older than the previous SSU rDNA estimate (310 mya) by Berbee and Taylor (2001). Estimations by Heckman et al. will be polyphasically discussed in the near future.

7.3 International Projects on Plectomycete Systematics and Genomics

7.3.1 ICPA and Its Activities

The International Commission on *Penicillium* and *Aspergillus* (ICPA) is a Commission of the Mycology Division of the International Union of Microbiological Societies (IUMS). According to the homepage of the Commission at the website (http://www.cbs.knaw.nl/CPA/ICPA.htm), the major aims are (a) to improve any or all aspects of the systematics of *Penicllium* and *Aspergillus*, (b) to improve the accuracy of *Penicillium* and *Aspergillus* identification in culture collections and in industrial applications, and (c) to assist the development of more effective taxonomic treatments of these genera. The activities of ICPA consisting of 13 specialists are very high. The past three international ICPA workshops were held in 1985, 1989, and 1997 in Baarn, The Netherlands. Their proceedings were published as the respective books demonstrating advances and trends in *Penicillium* and *Aspergillus* systematics (Samson and Pitt 1985; 1990; 2000).

7.3.2 The Fungal Genome Projects and Their Impacts

The genome of the eukaryotic model organism *Saccharomyces cerevisiae* was analyzed in 1996 (see http://genome-www.stanford.edu/Saccharomyces). The fungal genome project has started and selected *A. nidulans* as a target fungus; and gene sequences from the 4th chromosome has been sequenced from 1998 (Bennett 2001; http://www.genome.ou.edu/fungal.html). In Japan, the koji mold *A. oryzae* genome project has started in 1998, and the data of expressed sequence tag (EST) are available at the web site (http://www.aist.go.jp/RIODB/ffdb/index.html). Novel data from the fungal genome projects, including *Aspergillus* genomics (Bennett l.c.), cut out a new horizon through biotechnology as well as plectomycete systematics.

8 CONCLUSIONS

Molecular systematics and phylogenetics by the integrated analysis of phenotypic and genotypic characters have contributed better understanding of phylogenetic speculation, evolutionary relationships, and delineation of the plectomycete taxa that are generally characterized by cleistothecial or gymnothecial ascomata. The phylogenetic placement, which the loculoascomycete genus *Capronia* is basal to the plectomycetes, suggests that the cleositothecial and gymnothecial forms with irregularly distributed asci, both lacking forcible discharge in the plectomycetes, may have been derived from ascomata with bitunicate asci arranged in a hymenial layer having forcible ascospore discharge. As we have emphasized, the strictly anamorph (asexual, mitotic) species of *Aspergillus* and *Penicillium* and their relatives of economical importance are often recently derived from teleomorph (sexual, meiotic) lineages, and do not give any strong support to the existence of ancient anamorphic lineages. In addition, we have strongly suggested that strict anamorph taxa (e.g., *Geosmithia*, *Paecilomyces*, and *Sagenomella* spp.) based only on the morphological species concept should be phylogenetically reexamined and revised.

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Exploitation of GFP-Technology with Filamentous Fungi

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1 INTRODUCTION

The green fluorescent protein (GFP) has proved an outstanding tool for studying various biological questions and revolutionized functional cell biology. The jellyfish *Aequorea victoria* protein was introduced into cell biology and biotechnology shortly after the *gfp* gene was cloned (Prasher et al. 1992). It was shown that the gene could be expressed in other organisms and detected as green fluorescence following excitation with UV or blue light (Chalfie et al. 1994). Unlike GUS, expression and localization of GFP can be easily detected in living cells and without addition of cofactors, substrates, or other proteins (Sheen et al. 1995). The stability of the brightly fluorescing molecule allows visualizing molecular events *in situ* and in real time. Mutations of the *gfp*-gene have led subsequently to the design of a number of improved GFP varieties, which are not only brighter than the wild type, but also available in different colors, making multichannel detection possible [for review, see Chalfie and Kain (1998) and Tsien (1998)]. Multichannel detection was exploited, for example, in the field of microbial ecology, where differently marked bacteria strains could be followed simultaneously on plant roots (Bloemberg et al. 2000).

The versatility of GFP becomes obvious when considering that it can be expressed free under a constitutive (Lübeck et al. 2002; Mikkelsen et al. 2001) or specific promoter (Bowyer et al. 2000; Zeilinger et al. 1999), or in fusion to a nonfluorescent protein under either class of promoter. Thus, free expression of GFP typically answers the question: in which developmental stage, and in which cell type this is gene active? The expression of GFP-fusion proteins answers the questions as to where a protein is localized, whether it migrates and as to its function. Surprisingly, GFP seems to be nontoxic and biologically inert, since, according to the literature available, free GFP or GFP-fusion proteins rarely inhibit cell functions.

In the jellyfish *A. victoria*, GFP is excited by the blue chemiluminescence of the protein aequorin in a process called fluorescence resonance energy transfer (FRET) (Shimomura 1998). This process can be used to study protein–protein interactions *in vivo* with color varieties of GFP (Pollok and Heim 1999). There is a continuous development of novel GFP constructs which also include bioindicators, like the so-called cameleons for dynamic measurements of free Ca^{2+} in living cells (Fan et al. 1999) and pHluorins for the pH-value (Miesenbock et al. 1998). After summarizing the structure of GFP, discussing transformation strategies for fungi and outlining the techniques needed for GFP detection and visualization, the present article focuses on the application of GFP in fungal biotechnology [see also Lorang et al. (2001)].

2 THE PRINCIPAL FEATURES OF GFP

The GFP is a barrel-shaped protein the center of which carries the chromophore. The wall of the barrel is a β-sheet structure consisting of 11 antiparallel strands, which can explain the stability of the molecule (Philips 1998). The GFP is a molecule of 238 amino acids and the chromophore is formed by cyclization and oxidation of its own Ser–Tyr–Gly sequence at position 65–67 (Heim et al. 1994). The *gfp*-gene does not occur naturally in terrestrial organisms. It was soon realized that wild type GFP inserted into the genome of different procaryotes and eucaryotes yielded insufficient fluorescence for reliable detection. Therefore, molecular designing studies were initiated to enhance the fluorescence (Sheen et al. 1995). Mutations leading to changes in the amino acid composition of the chromophore and other modifications

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such as changes in codon use have resulted in new synthetic GFPs with enhanced brightness and decreased photobleaching [i.e., Tsien (1998)], as well as in GFPs which are targeted to specific cell organelles or modified in solubility (Fernándes-Ábalos et al. 1998; Mankin and Thompson 2001). By this approach, a number of distinct classes of GFP have been established (Tsien 1998). Variants such as blue (BFP), cyan (CFP), and yellow (YFP) that exhibit differences in excitation and emission spectra are now available for use in molecular biology. Indeed, many different *gfp*-vectors specifically designed for special use can now be purchased from various companies. A new gene, *DsRed*, encoding a similar chromophore to GFP, but with a unique far-red fluorescence, has been cloned from a coral belonging to the genus *Discosoma* (Fradkov et al. 2000). Furthermore, a gene coding for a chromophore in the sea anemone *Heteractis crispa* has been modified recently to code for a far-red fluorescent protein (Gurskaya et al. 2001). This new gene, *HcRed*, may also prove useful as a reporter gene in different organisms.

Wild type GFP does not, presumably, work efficiently in filamentous fungi (Fernández-Ábalos et al. 1998; Maor et al. 1998; Spellig et al. 1996). The main effort has concerned improvement/enhancement of GFP for use in mammalian and plant cells and indeed the first attempts to use the technology with filamentous fungi were unsuccessful (Maor et al. 1998). The first breakthrough came with the work of Spellig et al. (1996), who transformed *Ustilago maydis*, a fungal pathogen of maize, with a plasmid construction, the codon usage of which had been optimized for use in plants but with a promoter from a fungus belonging to Basidiomycota. Since then the GFP-technology has been further developed for use with other fungi for studying cell function, gene expression, and interactions with other organisms as well as for studying fungal ecology.

3 TRANSFORMATION OF FUNGI WITH GFP

Transformation of fungi is the main topic of the chapter by R. L. Mach, (see Chapter 10). In brief, the PEG transformation system has been used mostly for transforming fungi with *gfp*. This is normally achieved by cotransformation of fungal protoplasts with a vector carrying the *gfp* and a vector containing a selective maker gene such as a gene coding for, for example, hygromycin resistance [e.g., Mikkelsen et al. (2001)]. In some cases, the gene coding for the selective marker is inserted directly into the *gfp* vector instead (Lorang et al. 2001). Transformants can then be selected when protoplasts regenerate under selection pressure on agar to which, e.g., hygromycin is added. The PEG transformation works well with most fungi, which can germinate and grow on agar (Lübeck et al. 2002; Zeilinger et al. 1999) and have also been used with some Oomycetes (Bottin et al. 1999; van West et al. 1999). Subculturing of hyphal tips or single spore transfers must, however, often be carried out several times to obtain stable transformants (Lübeck et al. 2002; Mikkelsen et al. 2001), especially when multinucleate protoplasts have been used in the transformation (Lorang et al. 2001). With biotrophic fungi, it might be necessary to adapt other transformation procedures. Particle gun transformation was used in the case of powdery mildew fungus *Blumeria graminis* growing on barley leaves (Christiansen, personal communication). The bombardment is carried out as soon as the basal cell from which conidia will develop is formed. Selection of conidia expressing GFP is carried out under a fluorescence dissection microscope and a special procedure for transferring GFP expressing conidia to new leaves by use of a curette (a dentist tool) has been developed. Problems with transient expression were experienced and GFP expression in germinating spores stopped at the stage of appressorial formation although the GFP expression should have been constitutive. In trying to overcome these problems, they made a single construct containing both *gfp* and the *bar* gene which confer resistance to the herbicide Basta (Christiansen, personal communication). Bombardment of the fungus was carried out on Basta resistant leaves, which were then transferred to a selective medium containing the herbicide. By this means stable tranformants were obtained expressing *gfp*, which persisted to subsequent spore generations on plants. Work is in progress for developing a similar transformation system on plants based on hygromycin selection (Christiansen, personal communication). Development of Agrobacterium-mediated transformation methods for transforming powdery mildew on barley with *gfp* also seems promising (Christiansen, personal communication). However, if used for studying gene expression in fungi or plants it might be necessary to use a gene construct, which cannot be expressed in *Agrobacterium*. This was the case with the intron-containing gene constructs made by Mankin and Thompson (2001), which allowed plant cell GFP expression in the presence of the bacteria.

Spellig et al. (1996) used the *sGFP* (*Ser65T*) gene in which serine was replaced with threonine at amino acid 65 (Chiu et al. 1996; Heim et al. 1994; 1995) This synthetic GFP belongs to class 2 according to Tsien (1998), often are referred to as red shifting GFPs. The gene had been modified in codon usage to improve its adaptation to mammalian cells (Haas et al. 1996). However, these modifications in codon usage also improved its practicability in plants (Chiu et al. 1996). The modifications made of the gene sequences tend to increase the overall GC content of the transcript, which might improve stability of GFP in these organisms (Fernández-Ábalos et al. 1998). A cryptic intron splice found in wild type *gfp*, which can reduce GFP expression in plants was also deleted by the codon optimization. Apparently, the changes in sequences leading to modification in codon usage were also needed for expression in *U. maydis*. Comparative studies of different forms of the synthetic *gfp* having the S65T mutation and regulated by the same promoter, indicate that optimization of codon usage might be important for enhancing GFP expression in fungi, but results are too sparse to be conclusive (Fernández-Ábalos et al. 1998). Other specific requirements for obtaining fluorescence can be

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necessary in some fungi. This was demonstrated in the fungus *Schizophyllum commune* where insertion of an intron after the SGFP stop codon was required to obtain the correct transcription of this gene (Lugones et al. 1999).

Since the *gfp* variant exhibiting the S65T mutation was used successfully with a fungus by Spellig et al. (1996), it has formed the basis for constructing GFP expression vectors, which have been used for transforming fungi from Basidiomycota, Ascomycota (Lorang et al. 2001), Deuteromycota (Lübeck et al. 2002; Zeilinger et al. 1999), and Zygomycota (Schilde et al. 2001) as well as the Oomycota (Bottin et al. 1999). Thus, the gene blue-*SGFP-TYG* referred to as *SGFP* has been used most frequently for transformation of filamentous fungi [see Table 1 in Lorang et al. (2001)]. Especially the vector construction pCT74 made by Lorang et al. (2001) has proven useful for transforming a range of fungi from different genera with *gfp*.

Different promoters can drive the gene. Attempts to use the same construct as Spellig et al. (1996) for transforming different fungi belonging to Ascomycota were unsuccessful and this was attributed to the use of a promoter from *U. maydis* (Maor et al. 1998). The *gpd* promoter from *Aspergillus nidulans* has been used in transformations of many different fungi providing high levels of constitutive gene expression including GFP expression (Green and Jensen 1995; Lübeck et al. 2002) and the *ToxA* gene promoter from *Pyrenophora tritici-repensis* also drives high level of constitutive GFP expression in many Ascomycetes (Lorang et al. 2001). Two promoters cloned from the Oomycete *Bremia lactucae* were successfully used in transformation of other Oomycetes, *Phytophthora parasitica* var. *nicotianae* (Bottin et al. 1999) and *P. palmivora* (West et al. 1999) with *gfp*. Other promoters have been chosen because they regulate the expression of specific genes of interest. Thus, it is recommended to use promoters from the same fungal species, which is being transformed, or from fungi related to the transformant in order to obtain good expression of GFP. The choice of promoter, of course, also depends on the scientific question posed in the experiments where the technology is being used.

4 DETECTION TECHNIQUES USED WITH GFP

The GFP varieties are fluorochromes. Like all fluorochromes they absorb photons at a specific wavelength (excitation peak) and emit photons with a longer wavelength (emission peak). It is possible to screen for GFP-fluorescence macroscopically just by using a UV- or blue light hand lamp and an appropriate barrier filter for eyes or camera that only transmit light of the appropriate emission wavelength (see Table 1). However, since the eyes are not very sensitive to small differences in fluorescence intensity, a more objective measuring device is absolutely preferable. Another problem common for

Table 1 Fluorescence properties of the most important fluorescent protein varieties and filter settings

Fluorochrome	E BFP	E CFP	E GFP	E YFP	DsRed	HcRed
Color	Blue	Cyan	Green	Yellow	Red	Red
Excitation light	UV	Violet	Blue	Green	Green	Yellow
Excitation peak	382	430	489, 498	513, 520	556	588
Emission peak	448	476	507, 516	527, 532	583	614
Dissection microscope						
Excitation	360/40	425/40	470/40	(480/40)	546/10	—
Beam splitter	Not needed with extra excitation light path, otherwise see fluorescence microscope					
Emission	420 LP	475 LP	525/50	(515 LP)	590 LP	—
Fluorescence microscope						
Excitation filter	390/22	436/10	475/40	(480/40)	546/12	560/40
Beam splitter	420	460	500	(505)	560	595
Emission filter	440/50	470	530/25	(527/30)	575–640	645/75
CLSM						
Laser line	351/364	458	488	514	543/568	(543)/568
Beam splitter	See beam splitters for fluorescence microscope or use dynamic beam splitter					
Emission setting	See filters for fluorescence microscopy or use spectrometric filtering					
Two-photon microscope						
Laser tuning	780–810	780–810	860–960	860–960	—	—
Beam splitter	Fixed infrared beam splitter					
Emission setting	See filters for fluorescence microscopy or use spectrometric filtering					

Wavelength values (in nm) given are examples, others filter combinations offered by the microscope manufacturers might be more selective. Double and triple filter cubes as necessary for two or three channel detection are not listed. Some CLSMs have special options for spectral selection of emission light ("dynamic beam splitter" and "spectrometric filtering"): These options allow to adjusting detection precisely to the fluorescent-protein variety in question. LP, long pass filter; values in brackets, filter combination not ideal.

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the detection of a fluorochrome is the autofluorescence arising from endogenous sources, like phenols, tannic acids, cell wall compounds, and others. The choice of selective band-pass filters excluding autofluorescence, and the choice of a GFP-variety that does not overlap in excitation or emission wavelengths with autofluorescence in the sample is of high importance.

4.1 Dissection Microscope Equipped with Fluorescence Illumination

A dissection microscope (stereomicroscope) can be used for screening of petri dishes, containing cultures of yeast, bacteria, filamentous fungi or seeds, and small plants. It has to be equipped with epifluorescence illumination and a filter cube allowing selection for the wavelength of the respective GFP-variety. Epifluorescence illumination uses incident light (typically from a mercury lamp) that is coupled by a beam splitter or through an extra beam path into the objective, which focuses it onto the specimen. The emitted fluorescence passes the objective, beam splitter, and a specific barrier filter and can either be observed directly or documented with a conventional or CCD-camera. Fluorescence is often weak, thus pushed films or high-sensitivity cameras with Peltier cooling element are preferable.

Highly expressing cells are easily recognized and documented. However, small differences in fluorescence (e.g., when GFP-expression is under a weak promoter) are nearly invisible for the human eye. Therefore, it should be possible to set the exposure of the film or CCD-camera to fixed standard values. After recording wildtype cells (without GFP) and transformed ones with the same settings, the specific GFP-fluorescence will stand out.

4.2 Fluorescence Microscopy

Conventional fluorescence microscopes are valuable to localize GFP-fluorescence to single cells and even cell compartments. In comparison to confocal laser scanning microscopes (CLSM), they are much cheaper, easier to use, and not so time-consuming. Epifluorescence technique (see earlier) with incident illumination is state of the art for fluorescence microscopy for more than two decades. Fluorescence microscopes are mostly equipped with mercury or xenon lamps. Halogen lamps are, in general, too weak for effective fluorescence microscopy.

Again, the choice of the filter combinations sitting in filter cubes is decisive. It should be possible to change the filter combinations without screws (filter wheel), and the single excitation filters, beam splitters, and barrier filters should be replaceable in case that a more appropriate combination for the GFP in question is available. Microscope and filter manufactures offer a number of double- and triple-color filter combinations that can be used for visualization of two or three different fluorochromes at the same time. Objective lenses should be selected after their numerical aperture (NA), as higher the value as more light is collected and as higher is the resolution. The magnification factor itself is negligible for the demands of fluorescence microscopy. Because of the role of the NA, dry lenses are less suited for critical applications than immersion lenses. Today, the choice of monochromatic or color CCD-cameras with and without cooling for documentation is large. Although digital photography is certainly about to replace the conventional film-bound photography, it should be noted that the quality of well-exposed color slides is still difficult to match with digital techniques.

For advanced techniques like FRET and other ratio imaging methods, a monochromator between the light source and appropriate image processing software is necessary.

4.3 Confocal Laser Scanning Microscopy

The advent of GFP as fluorochromes also meant a revolution for confocal microscopy. Because of its brightness and stability, GFP is in a way an ideal fluorochrome for this technique. The CLSM technique is an extension of conventional epifluorescence microscopy. A light spot from a laser source is focused to a single 3-D point in the specimen. Fluorescence at this point is recorded by a highly sensitive photomultiplier. The laser beam is scanned in *x*/*y* direction through the specimen, so that a rectangular field is covered. The confocal adjustment of the aperture in front of the photomultiplier (detection pinhole) results in the removal of out-of focus fluorescence, which is an inherent problem in conventional fluorescence microscopy. Based on intensity changes during scanning, the CLSM software reconstructs an image which is actually an optical section of about 1 μm thickness, depending upon the NA of the objective used. The optical plane can be moved vertically (in *z*-direction) in the specimen and the penetration depth is up to 100 μm from the surface, allowing in many cases a direct observation of living cells without any preparatory dissection.

The manufactures of CLSMs offer today microscopes with multichannel detection and filter or alternative techniques to select appropriate excitation and detection wavelength ranges. In comparison to a xenon or mercury lamp there is a limitation in excitation wavelengths, and the commercial available laser sources are not 100% fitting to the absorption maxima of the different GFP-varieties (see Table 1).

However, when using the green GFP-varieties, the 488 nm line of the He/Ar laser present in most CLSMs fits nicely and gives rise to clear optical sections.

Confocal laser scanning microscopy with laser sources implies image processing; it is not possible to see confocal images in the eyepieces. Only confocal microscopes with so-called Nipkow-discs and conventional light sources make it possible to see the optical sections directly and in real time. Depending on the pixel resolution, scanning of the laser beam through the image field takes something between 0.1 and 1 s. Accordingly, the CLSM "writes" between 1 and 10 frames/s.

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In comparison to conventional epifluorescence microscopy, the indirect observation of the image and the selection of 1 μm optical sections means that recording of images with CLSM is much more time consuming, though the result in most cases is much better in sharpness, contrast, and subcellular resolution.

The inbuilt imaging software in most CLSM is already set up for recording of time series and 3-D reconstructions and is prepared for advanced ratio imaging techniques like FRET and measurement of bioindicator molecules.

An interesting development in microscopic techniques relevant for GFP-detection is two- or multiphoton microscopy. This is either an accessory for CLSMs or can be a stand-alone instrument. Instead of using confocal apertures, a two-photon microscope makes use of pulses of infrared lasers of high intensity. The excitation wavelength is roughly double the absorption wavelength of the fluorochrome. Fluorescence is evoked by the femto-second event that two photons hit one molecule. Since, this happens only in the focal plane, basically no out-of focus fluorescence is excited. The infrared laser beam is able to penetrate deeper into the specimen and is less damaging to cells. Moreover, the fluorochrome is bleached only in the very focal plane. Since, the infrared lasers available for two-photon microscopy are tunable, a great range of fluorochromes can be excited, including most of the GFP varieties (see Table 1).

5 GFP USED FOR STUDYING CELL FUNCTION IN FILAMENTOUS FUNGI

Some of the constructs for transforming *A. nidulans* made by Fernándes-Ábalos et al. (1998) were designed to produce soluble GFP. The result was that the soluble GFP could be found throughout the cytoplasm and entered the nucleus in transformants. The GFP was excluded from vacuoles and mitochondria although these organelles were present in the mycelium. These results were confirmed by using immunogold staining of GFP in the hyphae (Fernándes-Ábalos et al. 1998).

Green fluorescent protein can also be used for following organelles in the cell. Fusion, for example, of GFP to the GAL4 DNA-binding domain will target GFP to the nucleus (the fusion protein is targeted to the nucleoplasm and the spindle plaque) as demonstrated in *A. nidulans* (Fernándes-Ábalos et al. 1998). Nuclear division has been followed in *A. nidulans* by this technique and new information about the different phases in mitosis has been obtained (Fernándes-Ábalos et al. 1998). Genes important for nucleus migration have also been revealed by targeting GFP to nuclei in *A. nidulans* mutants affected in genes believed to be important for migration (Suelmann et al. 1998). These events can be studied in time course studies and are presented, for example, as video clips on the Internet (see http://www.unimarburg.de/mpi/movies/movies.html and www.uni-marburg.de/mpi/movies/apsbnew.mov). The GFP can also be targeted to the endoplasmatic reticulum by cloning ER retention signals into a vector used for fungal transformation with *gfp*. This was indicated in *A. nidulans* where GFP was localized in the cells exhibiting a similar pattern as ER in plant cells (Fernándes-Ábalos et al. 1998). A similar strategy for targeting and then following movement of mitochondria in *A. nidulans* has also been successful (Suelmann and Fisher 2000).

Khalaj et al. (2001) used a translational fusion of GFP to the protein glucoamylase for studying the secretion pathway in *A. niger*, which still is not understood very well. The fungus is exploited commercially for the production of heterologous proteins because of its secretory capacity. Khalaj et al. (2001) studied the effect of using different protein transport and cytoskeletal inhibitors on a transformant expressing the GFP-fusion protein mentioned earlier. The fluorescent protein could be monitored both in space and time by fluorescent microscopy following treatments with inhibitors. In this way, new information about the endomembrane system in the fungus was gained. Treatment, for example, with the inhibitor brefeldin A (BFA) initially revealed a fluorescent ER network throughout the hyphae and also accumulation of the fusion protein around the nuclei. Later fluorescent vesicle-like bodies, consisting mainly of vacuoles, replaced the reticulate network and after about 8 h larger vesicles containing GFP were observed. These large vacuoles were seldom seen in untreated hyphae and when removing the treated hyphae from BFA it restored a phenotype identical to that seen before the treatment (Khalaj et al. 2001).

6 GFP IN FUNGAL ECOLOGY AND PLANT PATHOLOGY

6.1 Visualization *in Situ*

Reporter technology where filamentous fungi are transformed with constitutive expressed genes, which enables monitoring the fungus in the environment where they live, have contributed with important information about fungal ecology and their interactions in the environment. One of the systems which is used mostly with fungi that allows for *in situ* monitoring is the GUS-system based on the β-glucuronidase reporter gene (*gus*). This has successfully been used in the study of plant pathogen interactions (Eparvier and Alabouvette 1994) and in ecological studies of fungal biocontrol agents (BCAs) including quantification of fungal growth and activity in the root zone (Green and Jensen 1995; Green et al. 2001). It is a prerequisite for success that it is used only in systems exhibiting a low background glucuronidase (GUS) activity. When used in natural soil, for example, problems with a high GUS background are likely due to indigenous microorganisms capable of producing the enzyme (Jensen et al. unpublished results). The method requires addition of substrates and a period for incubation. Problems can emerge especially during visualization due to insufficient penetration of the substrate into the sample such as plant leaves. Another problem can rise if the blue product leaks out

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from the transformant during incubation and results in unspecific staining (Green et al. 2001). Thus, the GUS system may be of limited usefulness in fungal ecology studies although it has proved very useful especially in quantitative studies of fungal activity (Green and Jensen 1995; Green et al. 2001).

One of the advantages of GFP is that no exogenous substrate needs to be added for detection. Only excitation light and oxygen is needed. Spellig et al. (1996) used SGFP fused to promoters from *U. maydis* with different regulatory characteristics. Firstly, they demonstrated that GFP could be expressed constitutive both in the haploid yeast-like stage and in the infectious dikaryon stage with filamentous growth. Subsequently, in their studies of the fungus in infected maize plants, they concluded that GFP did not affect the development of *U. maydis* in plants and that GFP was useful as a reporter of different developmental stages of the fungus in the host plant including spore formation (Spellig et al. 1996). Background fluorescence was not a problem as the fungus developed primarily outside wounded plant tissue, which exhibited a green autofluorescence with a spectrum similar to that of GFP. Indeed, background fluorescence can, instead of being a problem, be useful for localization of the GFP marked fungus in leaf tissue both when using epifluorescence microscopy and CLSM for detection (Jensen et al. unpublished results; Rohel et al. 2001b).

Trichoderma harzianum is one of the most used species in BCAs for biological control of plant diseases (Jensen and Lumsden 1999). It is important to have methods for monitoring BCAs and study their ecology following their application to soil or plants, in order to ensure optimal biocontrol efficacy. Bae and Knudsen (2000) marked a strain of *T. harzianum* with both *gfp* and *gus* reporter genes and used this transformant for autecology studies in soil. The antagonistic fungus was formulated as alginate pellets and its growth from a pellet into natural soil was studied. Conidia, conidial germination, and mycelia growth could be observed in the soil within two days. After 5 days most structures consisted of chlamydospores in which the GFP intensity was too low for clear observation, but GFP in hyphae could still be observed after 10 days (Bae and Knudsen 2000). Their results also indicated that the growth was more restricted in natural soil compared to sterile soil; this can probably be attributed to competition from the indigenous microflora. However, the transformant they had selected also grew more slowly than the wild type, which made it difficult to obtain conclusive results, as the transformant was not studied in sterile soil. It must be emphasized that transformants must be tested to be similar to the wild type if they are to be used in ecological studies. Bae and Knudsen (2000) also used the transformant of *T. harzianum* for studying colonization of sclerotia of the pathogen *Sclerotinia sclerotiorum* in soil, but it is not clear whether it was GFP or GUS, which was the useful reporter in these studies.

Infection and colonization of maize leaves by the pathogen *Cocholiobolus heterostropus* (causing Southern corn blight) was studied by Maor et al. (1998). They made their own vector construction for transforming the pathogen with *SGFP* and with constitutive GFP expression. They were able to observe spore germination and follow growth of the fungus both on and inside leaves over time. The fungus could be visualized in infected leaves without clearing the tissue from the first infections to the end of disease development. The mycelium was found to be arranged in bundles growing parallel to the veins and was sometimes restricted to certain areas in the mesophyll (Maor et al. 1998). Quantification of GFP using serology correlated with fluorescence intensity and, therefore, Maor et al. (1998) suggest that this can be used for estimating fungal biomass and for disease indexing for this disease.

We have marked a near commercial biocontrol strain *Clonostachys rosea* IK726 with *gfp* for use in ecology studies (Lübeck et al. 2002). The SGFP-construct of Maor et al. (1998) was used. This GFP transformant can be visualized with epifluorescence microscopy on plant surfaces such as on leaves, roots, and seeds as well as in soil or in growth media (sphagnum based compost and vermiculite) used in greenhouses (Lübeck et al. 2002). Conidia, spore germination, mycelial growth, and conidiophores could be visualized. Background fluorescence was troublesome in some cases, but the problems could normally be reduced to a minimum with the right choice of excitation and barrier filters (Jensen, unpublished). Results from using CLSM on soil samples and on samples from growth media inoculated with the GFP mutant of *C. rosea* showed that the fungus can be visualized easily and that background fluorescence in the samples can be exploited to form 3-D computer images of the soil or growth substrate with the GFP mutant (Jensen unpublished; Lübeck et al. 2002). Recently, it has also been demonstrated that the GFP reporter is useful for following colonization of carrot seeds by *C. rosea* during seed priming—a traditional seed technology used with carrot seeds. *C. rosea* was inoculated to the water used for seed priming and spot samples of seeds were taken throughout the priming process and observed under the fluorescence dissection microscope. In this way, it was possible to optimize the colonization of the seeds by the antagonist during seed priming (Jensen et al. unpublished).

Mikkelsen et al. (2001) transformed the perennial ryegrass endophyte *Neotyphodium lolii* with *SGFP* using the same vector as Maor et al. (1998) and obtained a transformant with bright constitutive GFP expression. The infection in ryegrass could be followed and the mycelium could clearly be visualized in the leaf sheaths of *Lolium perenne* using epifluorescence microscopy (Mikkelsen et al. 2001).

Lagopodi et al. (2002) gained new information about how *Fusarium oxysporum* f.sp. *radis lycopersici* colonizes and infects tomato roots by use of CLSM and a GFP transformant of the pathogen. The CLSM has also been used in studying tomato root colonization by *F. oxysporum* f.sp. *radis lycopersici* and the bacteria *Pseudomonas fluorescens*. The fungus was marked with GFP and the bacteria with DsRed (Lugtenberg et al. 2001). This demonstrates the great potential for using microorganisms with different fluorescent reporters for studying microbial ecology in relation to

biological disease control, although there still is a need for more fluorescent reporters that can be used in fungi.

The GFP can also be exploited in combination with other fluorescent staining procedures. Henriksen et al. (1999), for example, combined GFP and staining with calcofluor white for studying growth and morphology of *A. niger*. In this way, they were able to quantify the relative area stained with GFP compared to the total area of the fungus stained with calcofluor white.

Different formae speciales of *F. oxysporum* (either pathogenic to strawberry, tomato, or melon) were compared for their ability to colonize the roots and cause disease symptoms on melon seedlings (Nonomura et al. 2001). A special incubation plate was designed for growth and *in situ* observation of the roots following artificial inoculation with *gfp* transformants of the different *Fusarium* spp. Monitoring the GFP transformants revealed that germinating conidia of both pathogenic and nonpathogenic strains were adhering to the root surface and revealed that the hyphae could grow on the root surface. However, only the pathogen could infect vascular tissue resulting in wilting of the host (Nonomura et al. 2001). These results agree with Eparvier and Alabouvette (1994) who used the GUS reporter for studying colonization of melon roots by pathogenic and nonpathogenic *F. oxysporum*.

The GFP has also proved useful for studying conditions such as xylose repression and maltose induction in either batch cultures or continuous cultivation of *A. niger*. The GFP was under the control of a glucoamylase promoter that is repressed by xylose and induced by starch and maltose. However, as it took 3–6 h before GFP could be detected following shift of the carbon source to inducing conditions and, as the stability of GFP is high, the method will have some limitations for studying fast changes in promoter activity (Henriksen et al. 1999). Such considerations are of course also relevant in using the GFP technology for studying promoter regulation in other systems. The GFP production could be followed both at steady state and under dynamic conditions and the changes in fungal growth and morphology could be described by image analyses on spot samples taken from the bioreactors (Henriksen et al. 1999).

The entomopathogenic fungus *Paecilomyces fumosoroseus* has also been marked with GFP (Cantone and Vandenberg 1999). Larvae of the diamondback moth *Plutella xylostella* were dipped in a suspension of the transformant and the fungus monitored throughout the infection process in the larvae. Fluorescent spores and hyphae were observed on the surface of the larvae and after 2 days the spores germinated, formed appressoria and penetrated the cuticle. Blastospores expressing GFP were also detected in the hemolymph of the larvae (Cantone and Vandenberg 1999). Adults of the Russian wheat aphid, *Diuraphis noxia*, were found to be contaminated on their antennae and forelegs by spores of the GFP marked strain of *P. fumosoroseus*, when they were exposed to sporulating cadavers for 8 h. The fungus proliferated on the surface of the adults, and the nymphs produced later died. Growing fluorescent hyphae and conidia formation could be observed on the dead nymphs (Cantone and Vandenberg 1999).

Both hyphae, sporangia and swimming zoospores could be visualized with GFP technology in the Oomycete plant pathogen *P. palmivora* (West et al. 1999). A constitutive expressed GFP reporter was applied and compared with the use of GUS as a reporter in *P. palmivora*. The GFP was concluded to be superior to GUS for visualization at the microscopic stage. Also, in comparison, it would be impossible to use the GUS reporter with fragile swimming zoospores, since the staining procedure is lethal (West et al. 1999). A few months earlier Bottin et al. (1999) published the first paper on the application of the GFP reporter with an Oomycete and showed similar results concerning GFP expression in the various fungal structures as described by West et al. (1999). They used a promoter from *B. lactucea* regulated by heat in transforming *P. parasitica* var. *nicotianae* (Bottin et al. 1999). It was clearly demonstrated that the promoter was upregulated by heat chock reflected as increased GFP expression. Although there was some trouble with tandem insertions they concluded that GFP could be used as a quantitative reporter for gene regulation in an organism belonging to Oomycota.

6.2 Plant Pathogen Interactions

Isocitrate lyase (ICL) is an important enzyme in carbon metabolism in the glyoxylate bypass and plays a role in gluconeogenesis. The gene is expressed during growth on two-carbon or fatty acid based substrates and is down regulated during growth on glucose. Thus, the promoter is repressed during normal glycolysis and TCA cycle metabolism (Bowyer et al. 2000). Regulation of ICL was studied in the plant pathogenic fungus *Tapesia yallundae* during infection of wheat. The ICL promoter from *Neurospora crassa* was used for fusion with *gfp* and transformed into the pathogen. The GFP expression was determined as fluorescence relative to a GFP standard curve based on fluorescence of pure GFP protein and given as percent of total protein, following test growth on different C-sources. It is important (as mentioned earlier with the Oomycetes) that there only is one insertion of *gfp* in the transformant, when this method is used for quantification, as there can be problems with tandem insertions such as nonlinear GFP production or gene silencing. Bowyer and coworkers verified that the promoter was induced in response to growth on two-carbon substrates, repressed by hexose sugars and that the regulation in the pathogen was similar to the regulation in *N. crassa* (Bowyer et al. 2000). The pathogen was then followed by CLSM during plant infection. The GFP expression was only observed during growth on the plant surface, which indicated that two-carbon metabolism is important at this stage of pathogenesis (Bowyer et al. 2000).

Septoria blotch (named after the anamorph stage: *Septoria tritici*) in wheat is caused by the fungus *Mycosphaerella graminicola*. The first few weeks after infection of leaves,

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the fungus grows ectotrophicly and colonizes the intercellular spaces without causing symptoms. Then necrosis and sporulation occur. As the fungus does not form haustoria it is suggested that an interface between the hyphae and host plant cells is established with a function similar to haustoria in the first period of infection. Rohel et al. (2001b) also chose a carbon source-repressed promoter from the gene encoding ICL in *N. crassa* and fused it to GFP for transforming *M. graminicola*. Tests of the transformant in liquid culture indicated that the promoter behaved in the same way as in *N. crassa*. Thus, glucose, fructose, and saccarose repressed the promoter. Subsequent GFP expression (i.e., carbon repression) in the infection process revealed that repression took place from penetration until sporulation, where new spores in pycnidia were fluorescent (Rohel et al. 2001b). This indicates that the host fungal interface is established during endophytic growth of the pathogen before sporulation takes place. A transformant with a constitutive GFP expression was used as a control in localizing the fungus throughout the infection process (Rohel et al. 2001b). The effect of the strobilurin fungicide, azoxystrobin, on *M. graminicola* following infection of wheat was studied by Rohel et al. (2001a). Azoxystrobin is normally believed to be effective prior to infection. However, due to its mechanism of inhibiting the pathogen it could have a curative effect after penetration of the host. Thus, they applied the transformant with constitutive GFP expression mentioned earlier and looked at the number and size of infections 3 weeks after inoculation by use of epifluorescence microscopy. Spraying of plants with azoxystrobin in the recommended dose or half rate was carried out at various stages of the incubation period. Although the clearest effect on number and size of infections were found following treatment within 3 days after inoculation, they still observed a significant effect by spraying after the penetration had started also on the morphology of the pathogen growing in the leaf (Rohel et al. 2001a).

The objective of the work of Dumas et al. (1999) was to use GFP as a reporter for studying the regulation of a gene (*clpg2*) coding for endopolygalacturonase. This is an enzyme believed to be of important in the infection process between the pathogen *Colletotrichum lindemuthianum* and bean plants. They fused *SGFP* to the regulatory sequences located upstream to the coding sequence of the gene and showed that transcription of *gfp* in the transformant reflected transcription of the wild type *clpg2* gene. By detecting GFP during infection of bean plants and the accumulation of *clpg2* transcripts by use of RT-PCR, they could conclude that the endopolygalacturonase gene is activated in the early stages of infection supporting the hypothesis that the enzyme is involved in breaking down pectin during infection (Dumas et al. 1999).

Quite a different approach for exploiting GFP technology in studying plant pathogen interactions was applied by Shirasu et al. (1999). The *mlo* gene conveys a broad-spectrum durable resistance against powdery mildew in barley. However, presence of the Mlo protein expressed by the wild type *Mlo* gene is sufficient to restore susceptibility to the pathogen in *mlo* resistant genotypes. This wild type *Mlo* gene was translationally fused to GFP and introduced into epidermal cells of a *mlo* resistant genotype. The ability of the fungus to infect and sporulate from epidermal cells expressing GFP (i.e., Mlo protein) was studied. They could conclude that expression of Mlo is necessary and sufficient for complementing *mlo* resistance leading to compatible relationships in single epidermal cells between the pathogen and the otherwise *mlo* resistant host (Shirasu et al. 1999).

6.3 Interactions Between Fungi

Spellig et al. (1996) transformed a haploid strain of *U. maydis* with *SGFP* under the control of a promoter, which was strongly inducible by pheromones from the compatible strain. They found that GFP was expressed only when the compatible strain was present, that the proportion of induced cells increased over time and that brighter GFP expression was seen in conjugating cells, although induction preceded formation of conjugation tubes. However, in the dikaryon stage the hyphal tip cell remained fluorescent although the promoter is down regulated in cells heterozygous for the mating type b. This seems to reflect the stability of GFP protein in the cell. Thus, although they could conclude that GFP functioned as a reporter for gene induction in *U. maydis* a shorter turn over of GFP might be required for more detailed studies of transient gene expressions in fungal cells. Green fluorescent proteins with increased sensitivity to special bacteria cell proteases have been designed resulting in variants with shorter protein half lives but these will only function in bacteria (Andersen et al. 1998). However, constructs are available for use in eucaryotes from the firm CLONETECH that might work in filamentous fungi.

Trichoderma harzianum controls many plant pathogens through mycoparasitic interaction with the target fungal organism and it is believed that extracellular cell wall degrading enzymes produced by the antagonist play an important role in these interactions. Special attention has been given to chitinases (Haran et al. 1996; Harman et al. 1993). Zeilinger et al. (1999) studied chitinase gene expression during mycoparasitic interaction of *T. harzianum* with the pathogen *Rhizoctonia solani*. They fused GFP to the 5′-regulatory sequences of the genes from *T. harzianum* coding for the chitinolytic enzymes *N*-acetyl-β-D-glucosamidase (*nag1*) and an endochitinase (*ech42*), respectively. Several transformants were tested for induction of fluorescence by colloidal chitin. Two transformant were selected each with multicopy insertions resulting in strong light emission, as they also showed a similar induction pattern of GFP as the respective chitinase genes showed in the wild type determined by Northern analyses. These transformants were then used in confrontation assays with the host fungi. When the two fungi were 10 mm apart, there were no

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expression either of *nag1* or *ech42*, but when 5 mm apart *ech42*::GFP expression was observed and the growth of *R. solani* was arrested. Expression of *nag1*::GFP was first observed 4 h after contact between the two fungi. Thus, they concluded that the endochitinase was induced before physical contact with the host fungus. There might even be longer distance between the two fungi when the first induction takes place as both fungi grew 0.6 mm/h and there is a time lack of at least 4 h before the GFP fluorophore is formed according to Heim et al. (1994). Further studies indicated that induction of *ech42* was triggered by oligosaccharides released from chitin in the host cell wall by low constitutive activity of chitinases in *T. harzianum* (Zeilinger et al. 1999).

7 CONCLUSION

The increasing number of exciting original papers using GFP shows that there is a tremendous potential for exploiting GFP with filamentous fungi. In fungal cell biology, novel approaches are spanning studies from mitotic division via migration of organelles to the organization of the cytoskeleton. Gene regulation and gene expression of enzymes involved in secretory pathways have been analyzed with GFP, either fused to the enzyme in question or expressed under a relevant promoter. In fungal ecology, the GFP technology offers even more choices. Spore germination, mycelia growth, and development of fungal structures were visualized *in situ* and regulation of inducible fungal promoters studied while the fungus was interacting in its natural environment. The GFP has, moreover, proved useful for detailed studies of signals involved in interactions between fungi and intensified studies in this are envisaged in the future. In plant pathology, new perspectives have evolved using GFP in order to follow gene expression and pathogen dynamics at different stages of infection. We have briefly touched novel GFP applications not yet tested with filamentous fungi. Optimally designed by selective mutations, a large variety of *gfp*-genes and derivatives await to be inserted into the fungal genome that fluoresce with different colors, pH, and calcium sensitivity and targeting to different organelles. For visualization and measurement of these GFP varieties advanced microscopic equipment is optimized allowing for vital observation from whole organism level down to the subcellular level. We hope that the present chapter inspires to the exploitation of the entire repertoire of fluorescent proteins in cell biology and ecology of filamentous fungi and to visualize the decisive steps in fungus–fungus and fungus–plant interactions.

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Antifungal Drugs in Fungal Infections

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1 INTRODUCTION

Based on infection sites, fungal infections in humans are mainly classified into two categories: superficial and systemic infections. In other words, the infections can range from superficial, noninvasive diseases of normal children and adults to life-threatening systemic diseases of immunocompromised individuals. Pathogenic fungi, causing superficial infections inhabit preferably the keratinized tissues such as stratum corneum of the skin, nail, and hair. Among these, dermatophytosis or tinea infections of the skin (tinea pedis and corporis) caused by dermatophytes, cutaneous candidiasis caused by *Candida* spp., and tinea versicolor caused by *Malassezia furfur* are commonly found as superficial skin fungal infections, and these infections are generally not life-threatening. These infections are usually treated with topical antifungal agents such as tolnaftate, ciclopirox olamine, clotrimazole, bifonazole, amorolfine, terbinafine, and butenafine. To treat tinea pedis, for example, tolnaftate, ciclopirox olamine, and clotrimazole are usually applied two or three times a day for four or more weeks. Bifonazole is the first drug that is used by once-a-day treatment regimen mainly because of its good retention in the stratum corneum of the skin. Amorolfine, terbinafine, and butenafine have also proved good retention in the stratum corneum so that once-a-day treatment with each of them can be sufficient to exert potent antifungal activity. Recently, as an additional advance in antifungal chemotherapy, successful short-term treatment of tinea infections has been achieved by the use of topical terbinafine. In the case of tinea unguium or onychomycosis (fungal infection of the nails), which is hardly cured by topical antifungal agents, oral griseofulvin had mainly been used until oral terbinafine or oral itraconazole became available. In contrast to superficial fungal infections, systemic fungal infections can be seriously life threatening and can be associated with high mortality. The incidence of disseminated systemic infections has grown in the past two decades, at least in part, as a consequence of the increasing number of immunocompromised people worldwide such as patients with HIV infection, organ transplant recipients, and patients receiving cancer chemotherapy. The immunocompromised patients are confronted with risk of systemic infection from commensal and/or ubiquitous species, primarily *Candida* spp. and *Aspergillus* spp. To treat these systemic fungal infections, four groups of agents based on the chemical structures have mainly been used to date: the polyene, the fluoropyrimidine, the imidazole, and the triazole. The polyenes are prototypic antifungal agents including amphotericin B and its analogue nystatin. These agents are effective across a broad spectrum of pathogens, but their utility is tempered by inherent toxicity, particularly nephrotoxicity. The class of fluoropyrimidines includes 5-flucytosine (5-FC) that was originally discovered as a potential antitumor agent. 5-FC is a nucleoside analogue and exerts antifungal effect by disturbing nucleic acids metabolism in fungal cells. However, the major drawback of the agent is that it creates multiple opportunities for selection of resistance. Thus, the agent is rarely used as a monotherapy. Regarding imidazoles, ketoconazole and miconazole have been used for the treatment of systemic fungal infections. In the case of ketoconazole, although it is effective against a number of medically important fungi, it causes a number of toxic effects including hepatotoxicity. Other imidazoles including miconazole also cause systemic toxicity so that their principal use is generally restricted to the treatment of superficial infections in topical formulations. The triazoles are distinguished from the imidazoles by the presence of a third nitrogen atom in the core structure. Fluconazole and itraconazole have some utility in invasive fungal infections although fluconazole is poorly active against *Aspergillus* spp. and sometimes causes development of azole-resistant organisms, and oral itraconazole is poorly absorbed in humans. As described previously, all four classes of current

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antifungal agents for the treatment of systemic infections possess somewhat drawbacks in terms of side effects and/or drug resistance. Thus, the search for new agents for life-threatening systemic fungal infections is continued. This chapter covers the medically important antifungal drugs in terms of historical background, and current and prospective management of fungal infections including problems such as drug-resistance and side effects.

2 ANTIFUNGAL DRUGS—HISTORICAL BACKGROUND

Efforts have been made to discover desirable antifungal agents with selectivity to pathogenic fungi since the beginning of the twentieth century (Iwata 1994). It has long been known that sweat can inhibit fungal growth. In the 1930s, it was speculated that certain kinds of fatty acids in the sweat are effective against fungi. Indeed, as the components of the sweat, propionic acid, butyric acid, lactic acid, and ascorbic acid proved effective. Among the substances in this categorized group, undecylenic acid and its salts, especially propyleneglycol dipropionate, were found to have potent antifungal activity against dermatophytes in the 1970s. 5-Chlorosalicylanilide and 5,5′-dibromosalicyl as salicylic acid-derivatives were found to exhibit antidermatophyte activity around 1950. Coloring matters such as malachite green and gentian violet were frequently used for the treatment of dermatophytosis, cutaneous candidiasis, and tinea versicolor in 1950s–1960s. Generally, phenols possess both antibacterial and antifungal activities, and among them haloid phenols and bisphenols were found to have prominently potent antifungal activity in 1950s–1960s. In a group of sulfurated compounds, dithiocarbamate derivatives, especially naphtyl-*N*-methyl-(1-naphtyl) thiocarbamate and 2-dimethylamino-6-(2-diethylaminoethoxy) benzothiazole were found to exert potent antidermatophyte activity in the 1950s–1960s. Furthermore, three thiocarbamate derivatives, tolnaftate, tolciclate, and liranaftate were discovered (Carneri et al. 1976; Iwata et al. 1989; Noguchi et al. 1963) and are used as topical drugs for the treatment of dermatomycosis. In organic diamines, propamidine, pentamidine and stilbamidine were found to have antifungal activity around the 1950s, and 2-bromo-2-nitro-1-phenyl-1-propyl methyl ether as a stilbamidine-related compound proved clinically effective in tinea infections. Thiazole derivatives have been known to have antifungal activity since the 1940s, and 2-dimethylamino-6-(β-diethylaminoethoxy)-benzothiazole was used as the most effective derivative. Around the 1950s, great achievements were made in antifungal chemotherapy. One of them is a discovery of nystatin, a polyene derivative, isolated from *Streptomyces nourse*. Nystatin is probably the first drug exerting excellent therapeutic efficacy in human fungal infections, especially candidiasis. Following the discovery of nystatin, amphotericin B was isolated from *Streptomyces nodosus* in 1955. Although the agent has defects such as inherent toxicity to humans, it has been used for the treatment of variety of systemic fungal infections until now. In addition to the discovery of nystatin, a deserving special mention was made about chemotherapy of superficial fungal infections: a discovery of griseofulvin that was isolated from *Penicillium griseofulvum* in 1939. Almost twenty-five years after the discovery, therapeutic effectiveness of oral griseofulvin was clinically proved in patients with onychomycosis as well as in patients with dermatomycosis (Arievich et al. 1965; Kaden 1965). In 1956, a fluoropyrimidine derivative, 5-FC synthesized by Hoffmann-La Roche Inc., is used as an oral drug for the treatment of systemic fungal infections. Although 5-FC exhibits a narrow antifungal spectrum, it proved to have advantages such as high blood concentration after oral administration and relatively low toxicity. More attention should be drawn toward discovery of a series of imidazoles and triazoles. In 1969, three imidazole derivatives were discovered successively. Clotrimazole was discovered by Bayer A.G., and miconazole and econazole were by Janssen Pharmaceutica Inc. These imidazoles are used for the topical treatment of superficial fungal infections, and miconazole is used as an injection for the treatment of systemic infections. Isoconazole, ketoconazole, and sulconazole were then discovered from 1969 to 1981. Ketoconazole, the first oral imidazole, proved clinically effective for the patients with systemic fungal infections. Bifonazole was discovered by Bayer A.G., and the development as a topical drug for the treatment of superficial fungal infections started form 1982. Bifonazole is the first topical agent to be used by once-a-day treatment regimen because of not only its potent antifungal activity, but also good retention in the stratum corneum of the skin (Plempel et al. 1983). In a group of triazole derivatives, fluconazole and itraconazole were successively discovered in 1978 by Pfizer Pharmaceuticals Inc. and in 1980 by Janssen Pharmaceutica Inc, respectively. Itraconazole has proved effective for the treatment of subacute to chronic infections including the endemic mycoses and opportunistic infection with *Aspergillus* spp., fluconazole is used routinely for mucocutaneous and systemic candidiasis (Kauffman and Carver 1997). The other groups of antifungal agents consist of ciclopirox olamine, naftifine, terbinafine, butenafine, and amorolfine. Ciclopirox olamine, a pyridone derivative discovered by Hoechst A.G. (Dittimar and Lohaus 1973), are used for the topical treatment of dermatophytosis and cutaneous candidiasis. Allylamine derivatives, naftifine and terbinafine were both discovered and developed by Sandoz A.G. (Georgopoulos et al. 1981; Petranyi et al. 1981; Stüz and Petranyi 1984) as drugs for the topical treatment of superficial fungal infections, and terbinafine is also used as an oral drug especially for intractable tinea infections and onychomycosis. Butenafine, a benzylamine derivative, first synthesized by Kaken Pharmaceutical Co., Ltd. (Arika et al. 1990), is used for the topical treatment of tinea infections but not of

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cutaneous candidiasis. A morpholine derivative, amorolfine synthesized and developed by Hoffmann-La Roche Inc. (Polak 1983), is used for the topical treatment of superficial fungal infections.

3 PRESENT STATE OF ANTIFUNGAL DRUGS

3.1 Superficial Fungal Infections

Superficial fungal infections often induce characteristic lesions, and a combination of clinical observations and laboratory investigations such as culture study and microscopy that usually give an accurate diagnosis. Fungal infection of the skin, nails, and hair is usually caused by dermatophytes. To treat tinea infections of the skin, undecylenic acid, ciclopirox olamine, tolnaftate, and tolciclate have been topically used in formulations of creams, ointments, and/or solutions up to now. Undecylenic acid exerts *in vitro* and *in vivo* antidermatophye activities, but the fungicidal activity is weaker than that of imidazole derivatives, miconazole and clotrimazole, and thiocarbamate derivatives, tolciclate and tolnaftate (Loebenberg et al. 1980; Wahab et al. 1978). Ciclopirox olamine has a broad spectrum of antifungal activity (Dittimar and Lohaus 1973), and it is clinically effective in treating dermatophytosis and cutaneous candidiasis. Although Sakurai et al. (1978) reported that ciclopirox olamine blocks transmembrane transport of radiolabeled leucine in *C. albicans* and *Saccharomyces cerevisiae*, the antifungal mechanisms have not been fully clarified yet. Tolnaftate has a selective antifungal activity against dermatophytes such as *Trichophyton* spp., *Microsporum* spp. and *Epidermophyton floccosum* (Noguchi et al. 1963; Weinstein et al. 1965). The antifungal mode of action of tolnaftate is an inhibition of squalene epoxidase, an enzyme that converts squalene into squalene epoxide, in ergosterol synthesis pathway, resulting in ergosterol deficiency with accumulation of squalene in fungal cells (Morita and Nozawa 1985). Tolciclate, like tolnaftate, has a potent antifungal activity against dermatophytes (Carneri et al. 1976), and their antifungal mode of action is an inhibition of squalene epoxidase (Ryder et al. 1986). The tinea infections are also topically managed with imidazoles, allylamines, butenafine, or amorolfine in formulations of creams, ointments and/or solutions for 2–4 weeks: generally more than 80% of patients cured (Evans 1997; Noble et al. 1998; Piérard et al. 1996; Tschen et al. 1997). Both clotrimazole and bifonazole have a broad spectrum of antifungal activity against a wide variety of pathogenic fungi (Barug and Bastiaanse 1983; Plempel and Bartmann 1972; Plempel et al. 1983). Their primary antifungal action is an inhibition of sterol 14α-demethylase (CYP51A1) in ergosterol synthesis pathway, resulting in a depletion of ergosterol and corresponding accumulation of 14α-methylsterols in fungal cells (Berg et al. 1984; Niwano et al. 1999; Vanden Bossche 1985). Both Naftifine and terbinafine exert potent antifungal activity against dermatophytes, while their activity against *Candida* spp. is relatively weak (Georgopoulos et al. 1981; Petranyi et al. 1981; 1984). Butenafine, like naftifine and terbinafine, has a potent antifungal activity against dermatophytes but a relatively weak activity against *Candida* spp. (Maeda et al. 1991). As is the case with thiocarbamate derivatives, antifungal mode of action of the two allylamines and butenafine is an inhibition of squalene epoxidase in ergosterol synthesis pathway (Iwatani et al. 1993; Petranyi et al. 1984; Ryder 1985). Amorolfine has a broad spectrum of antifungal activity against wide variety of pathogenic fungi such as dermatophytes, yeastlike fungi, dimorphic fungi, and dematiaceous fungi (Martin et al. 1992; Polak 1983; Shadomy et al. 1984). Amorolfine is classified as an ergosterol biosynthesis inhibitor (EBI), like azoles, allylamines and thiocarbamates, but its antifungal targets are inhibitions of Δ^{14} reductase and $\Delta^{8} \rightarrow \Delta^{7}$ isomerase in ergosterol synthesis pathway (Polak-Wyss et al. 1985). Fungal ergosterol biosynthetic pathway and antifungal targets of EBIs are shown in Figure 1 (Vanden Bossche et al. 1990b). When topical treatment with these antifungal agents fails, an oral drug is required. Treatment for 1 week with oral itraconazole at doses of 200–450 mg/day or for 2–4 weeks with oral terbinafine at a dose of 250 mg/day is usually effective (Boonk et al. 1998; Farag et al. 1994; Gupta et al. 1997; Hickman 1996; Parent et al. 1994; Tausch et al. 1998). Most cases of tinea ungium and other fungal infections of the nails (onychomycosis) have long been orally treated with griseofulvin. Recently, however, these infections of the nails can be treated effectively with oral itraconazole or oral terbinafine. Terbinafine at dose of 250 mg/day for 6 or 12 weeks proved effective in treating fingernail or toenail onychomycosis, respectively, and these treatment regimens provide more than 80% in mycological cure rates (Goodfield 1992; van der Schroeff et al. 1992). Willemsen et al. (1992) reported that in the patients receiving itraconazole for 3 months at a dose of 100 or 200 mg daily, therapeutic concentration of itraconazole is detected in the nail 6–9 months after therapy is stopped. This has led to the establishment of pulse therapy: itraconazole capsules 400 mg/day are administered for only 1 week each month, repeated twice or three times for fingernail onychomycosis and three or four times for toenail onychomycosis (Chen et al. 1999; De Doncker et al. 1995; Warnock 1998). Among superficial *Candida* infections, vaginal, penile or cutaneous candidiasis respond well to topical treatment with nystatin or imidazoles (Meis and Verweij 2001). Oral candidiasis in immunocompromised patients can respond to topical imidazoles or amphotericin B oral suspension or nystatin oral suspension (Meis and Verweij 2001). However, in neutropenic cancer patients and patients with HIV infection, oral antifungal agents are preferred because treatment with topical agents is sometimes associated with a high relapse rate (Smith et al. 1991). Although oral fluconazole has proved effective in treating oral candidiasis, emergence of fluconazole-resistant *Candida* species

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has become a problem especially in the patients with HIV infection receiving a long-term administration of fluconazole (Baily et al. 1994; Ruhnke et al. 1994).

3.2 Systemic Fungal Infections

Unlike superficial fungal infections, systemic fungal infections are generally life-threatening to immunocompromised people such as patients with HIV infection, organ transplant recipients, and patients receiving cancer chemotherapy. Although the medical challenge of management for systemic fungal infections has been partially conducted by the prophylactic, empiric, and therapeutic use of antifungal drugs such as amphotericin B, 5-FC and a series of azoles (miconazole, ketoconazole, fluconazole and itraconazole), the treatment of infections with such drugs is not always satisfactory for long-term therapy in terms of efficacy,

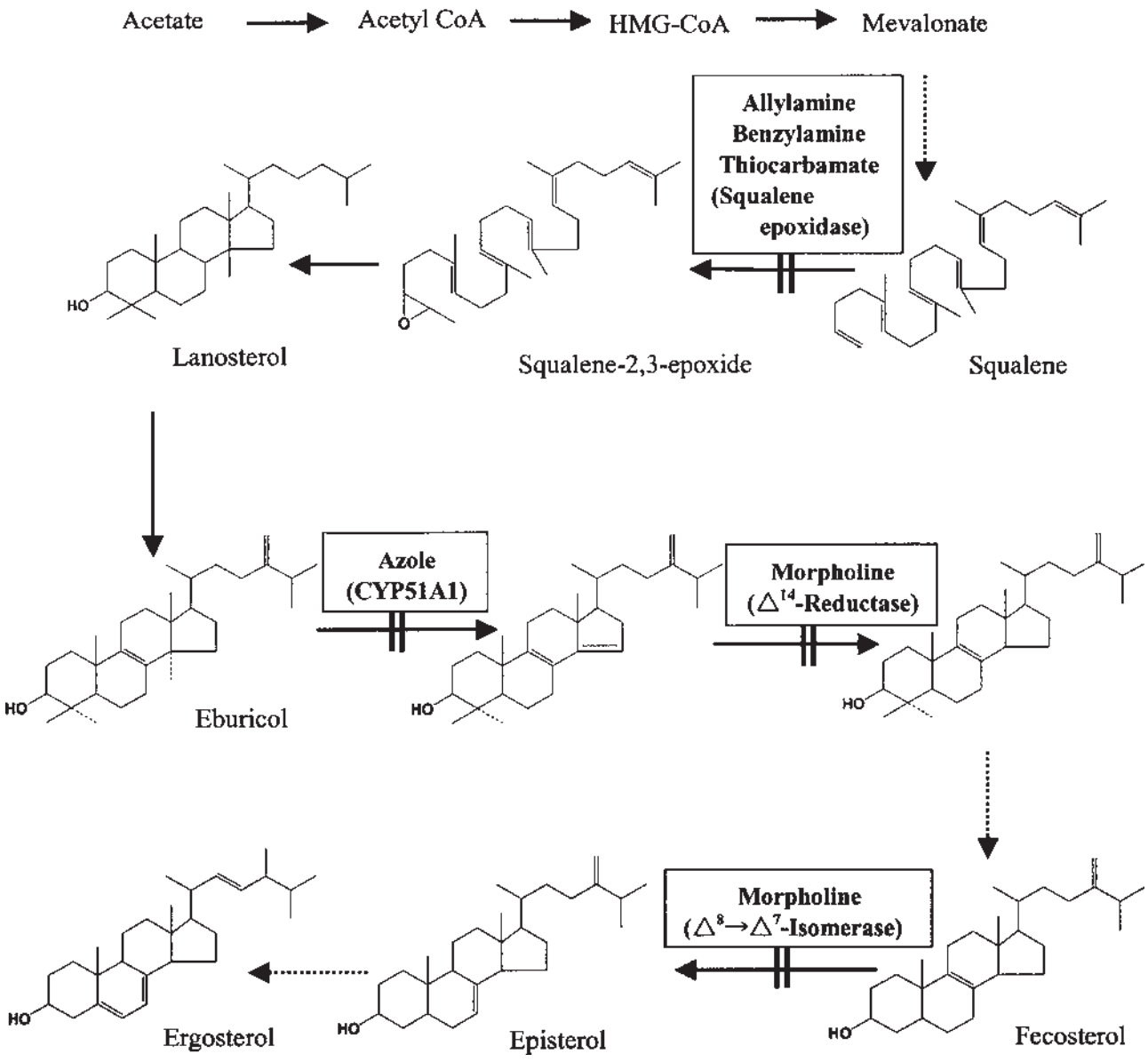

Figure 1 Antifungal targets of ergosterol biosynthesis inhibitors. Allylamines or benzylamines (such as terbinafine or butenafine) and thiocarbamates (such as tolnaftate) inhibit squalene epoxidase. Azoles (such as bifonazole and itraconazole) inhibit CYP51 A1 (sterol 14 α-demethylase). The morpholine derivative amorolfine inhibits Δ^{14}-reductase and $\Delta^{8} \rightarrow \Delta^{7}$ isomerase. Dotted arrows indicate multistep reaction. Source: Vanden Bossche et al. (1990b).

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toxicity, antifungal spectrum and emergence of drug-resistant fungal strains. Amphotericin B has a selective activity against fungal cells resulting from its preferential association with ergosterol-containing membranes (Kinsky 1961a,b; Lampen et al. 1960), and is a broad-spectrum and potent antifungal agent (Gold et al. 1956; Lechevalier 1960). The clinical use of amphotericin B is sometimes limited because of adverse reactions such as renal toxicity, hypokalemia and anemia (Maddux and Barriere 1980; Sabra and Branch 1990). 5-FC exerts its antifungal action by forming aberrant RNA and inhibiting DNA synthesis (Diasio et al. 1978). It has a relatively narrow antifungal spectrum, and especially exerts potent activity against *Candida* spp. and *Cryptococcus neoformans* (Holt and Newman 1973; Scholer 1970; Shadomy et al. 1970; Shadomy et al. 1973). 5-FC should not be administered as a single drug because of the frequent development of acquired drug resistance (Walsh and Pizzo 1988). Two imidazoles (miconazole and ketoconazole) and two triazoles (fluconazole and itraconazole) act as an EBI which target is sterol 14α-demethylase, CYP51 A1 (Vanden Bossche 1985; Vanden Bossche 1995), and have potent activity against a wide variety of pathogenic fungi (Corrado et al. 1982; Shadomy and Paxton 1976; Heel et al. 1982; Odds et al. 1986; Van Cutsem 1989). Miconazole and ketoconazole, however, have been largely supplanted by fluconazole and itraconazole in the treatment of serious fungal infections. Although both fluconazole and itraconazole are proved to be highly effective in laboratory animals, fluconazole is comparably less active against *Aspergillus* spp. than yeast-type fungi (Troke et al. 1987), and sometimes causes problematic development of azole-resistant organisms in the long-term therapy (Rex et al. 1995). Oral itraconazole is poorly absorbed in humans because of its high lipophilicity and low water solubility (Hardin et al. 1988). However, recent progress in antifungal chemotherapy has produced successful reformulation of the existing antifungal drugs (Finquelievich et al. 2000). The lipid-associated formulations of amphotericin B and cyclodextrin-associated formulations of itraconazole have recently been developed to overcome the disadvantages of these drugs. Three lipid-associated formulations of amphotericin B (amphotericin B-liposomal formulation, amphotericin B-lipid complex, and amphotericin B-colloidal dispersion) are available for intravenous use, and can protect toxicities caused by amphotericin B (Anaissie et al. 1998; Walsh et al. 1998; Walsh et al. 1999). In the case of itraconazole, two new formulations have been developed by using hydroxypropyl-β-cyclodextrin as a solubilizing excipient. De Beule and Van Gestel (2001) reported that the oral solution of itraconazole results in improved oral bioavailability not only in healthy volunteers but in a variety of groups of patients at risk of systemic fungal infections. Besides oral itraconazole solution, a new intravenous itraconazole formulation is also available for the patients who cannot take oral medication (Meis and Verweij 2001; Vandewoude et al. 1997). From a clinical viewpoint, most endemic mycoses such as blastomycosis, histoplasmosis, and coccidioidomycosis are treated with amphotericin B or itraconazole. Ketoconazole can also be effective for the treatment of immunocompetent patients with nonlife-threatening histoplasmosis, blastomycosis or paracoccidioidomycosis, but fluconazole is relatively less effective against endemic mycoses (Lortholary et al. 1999). The most common systemic fungal infections in immunocompromised patients are those caused by *Candida* spp. and *Aspergillus* spp. The various formulations of amphotericin B and itraconazole are effective for these systemic infections (Anaissie et al. 1998; Walsh et al. 1998; Walsh et al. 1999; Willems et al. 2001), but fluconazole is effective only for the infections caused by susceptible species of *Candida*, such as *C. albicans* and *C. parapsilosis* (Ikemoto 1989; Milatovic and Voss 1992; Presterl and Graninger 1994).

4 DRUG RESISTANCE IN FUNGI

Fungal resistance to antifungal agents has generally been less common than bacterial resistance to antibiotics. Recently, however, attention has been called to the fact that failures of treatment in fungal infections are somewhat attributable to the problem of antifungal resistance. Most of antifungal agents have a spectrum of activity excluding at least some genera of pathogenic fungi. For instance, griseofulvin is active only against dermatophytes so that other pathogenic genera such as *Candida* and *Aspergillus* can be called as "resistant or tolerant to griseofulvin." However, this is not the case of major impact of antifungal resistance, and a matter of concern revolves around emergence of antifungal-resistant individual species or strains within a normally susceptible fungus. Typical examples are resistance to 5-FC, which resembles most closely to the well-known situation in bacteria, and resistance to azole antifungal agents, which are growing with increasing use of the orally active azoles for a relatively long-term therapy (Rex et al. 1995; Whelan 1987). In the case of amphotericin B, resistance to the drug has been thought to be unusual among the common pathogenic fungi, but it is becoming a greater problem than has been supposed (Dick et al. 1980). This review describes the molecular mechanisms of resistance to 5-FC, azole antifungal drugs, and amphotericin B.

4.1 5-Flucytosine (5-FC)

Since resistance to 5-FC is well known and has been studied thoroughly, the mechanisms both of action and of resistance are understood (Vanden Bossche et al. 1994; Whelan 1987). After 5-FC is incorporated into fungal cells by a cytosine permiase, it is immediately deaminated to 5-fluorouracil (5-FU) by cytosine deaminase. The absence or low activity of the enzyme, cytosine deaminase, in mammalian cells is the basis for the low toxicity of the drug in humans. 5-FU is then converted by uridine monophosphate to 5-fluorouridylic acid, and is subsequently phosphorylated and incorporated into RNA, resulting aberrant RNA causes inhibition of fungal

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growth. In addition, 5-FU is converted to 5-fluorodeoxyuridine monophosphate, a potent inhibitor of thymidylate synthetase, which results in inhibition of DNA synthesis and nuclear division. Resistance to 5-FC can be derived from loss or mutation of any of the enzymes involved in its conversion and incorporation into RNA. Figure 2 summarizes antifungal mode of action of 5-FC. Some workers (Fasoli and Kerridge 1988; Kerridge et al. 1988) reported that a frequent clinical determinant of the resistance in the diploid *C. albicans* is a lesion in the UMP-pyrophosphorylase. Clinical problem is that the proportion of 5-FC-resistant cells can increase to the point that the population as a whole acquires resistant during treatment. Thus, monotherapy of 5-FC has been replaced by a combination therapy of amphotericin B and 5-FC since the combination exerts good synergy and reduces the emergence of secondary resistant isolates (Medoff et al. 1972).

4.2 Azoles

Among EBIs (Figure 1), the target enzyme of azole antifungal drugs is CYP51A1(sterol 14α-demethylase), one of the superfamily of cytochrome P450 and the products of *CYP51* gene (Vanden Bossche 1985; 1995). Until the late 1980s, acquired resistance to azole antifungals was rarely found. The first cases of resistance were reported in *C. albicans* from the patients receiving prolonged therapy with miconazole and ketoconazole (Holt and Azmi 1978; Horsburgh and Kirkpatrick 1983; Johnson et al. 1984). Furthermore, since fluconazole is used in a wide variety of clinical situations, antifungal resistance to the azole has been found more frequently. Several mechanisms by which yeastlike fungi such as *C. albicans* acquire resistance to azole antifungals have been reported: (a) cells fail to accumulate these compounds, (b) the affinity of CYP51A1 to these compounds is altered, (c) the content of CYP51A1 increases in cells, (d) the sterol $\Delta^{5,6}$ desaturase is inactivated, and (e) a combination of these mechanisms (Redding et al. 1994; Sanglard et al. 1995). As for the molecular mechanisms for the failure of resistant yeastlike fungi to accumulate azole compounds, multidrug efflux transporters of the ATP-binding cassette (ABC) superfamily and of the class of major facilitator superfamily (MFS) have been shown to be involved (Vanden Bossche et al. 1998). Sanglard et al. (1995) have shown that the ABC-transporter gene *CDR1* and the MF gene *BEN*^{R} (*CaMDR1*) were found to be over-expressed in resistant-isolates. Interestingly, they have also found that over-expression of *BEN*^{R} in *Saccharomyces cerevisiae* is involved in the specific resistance to fluconazole, but *CDR1* over-expression in *S. cerevisiae* conferred cross-resistance to different azole derivatives such as fluconazole, itraconazole, and ketoconazole. There are other multidrug efflux transporter genes of both classes found and cloned in *C. albicans* such as the ABC transporter genes *CDR2, CDR3* and the MF gene *FLU1* (Balan et al. 1997; Calabrese et al. 2000; Sanglard et al. 1997). Balan et al. (1997) have shown that *CDR2*, like *CDR1*, is over-expressed in *C. albicans* that are cross-resistant to azole derivatives. Figure 3 shows a schematic representation of these drug efflux systems in *C. albicans* (Niimi et al. 1997). The mechanism of azole resistance developed by the over-expression of multidrug efflux transporter genes is also found in other *Candida* species. Sanglard et al. (1999; 2001) showed that *C. glabrata* multidrug transporter genes (*CgCDR1* and *CgCDR2*), similar genes to the *C. albicans* ABC transporter *CDR* genes, are important mediators of resistance to azole derivatives in *C. glabrata*. Vanden Bossche et al. (1990a) reported that alterations of the affinity of CYP51A1 to azole antifungals are another important mechanisms by which azole resistance is developed. Lai and Kirsch (1989) have successfully obtained four separate mutations of *CYP51A1* gene from resistant *C. albicans* isolates: Tyr^{132} to His, Ser^{405} to Phe, Gly^{464} to Ser, and Arg^{467} to Lys. In addition, over-expression of *CYP51A1* has also been described as a resistance factor in a few *C. albicans* and *C. glabrata* isolates (Sanglard et al. 1995; Vanden Bossche et al. 1992). Other molecular mechanism by which azole resistance is developed is inactivation of sterol $\Delta^{5,6}$ desaturase in *C. albicans* isolates (Kelly et al. 1996; Nolte et al. 1997). In this case, these

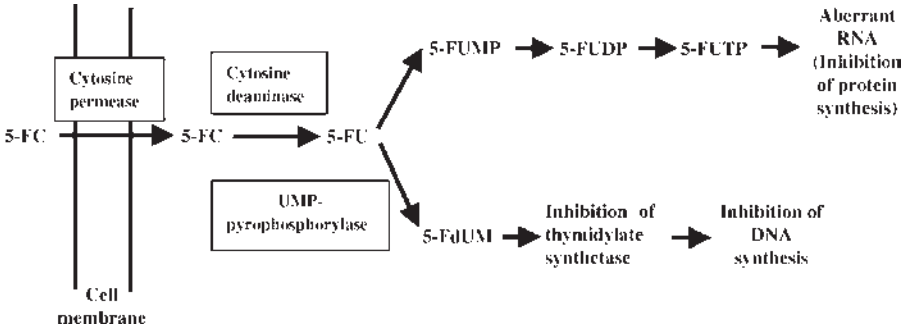

Figure 2 Antifungal mode of action of 5-flucytosine (5-FC). UMP, 5-FU, 5-FUMP, 5-FUDP, 5-FUTP, 5-FdUMP stand for uridine monophosphate, 5-fluorouracil, 5-fluorouridine monophosphate, 5-fluorouridine diphosphate, 5-fluorouridine triphosphate, 5-fluorodeoxyuridine monophosphate, respectively. Source: Vermes et al. (2000a).

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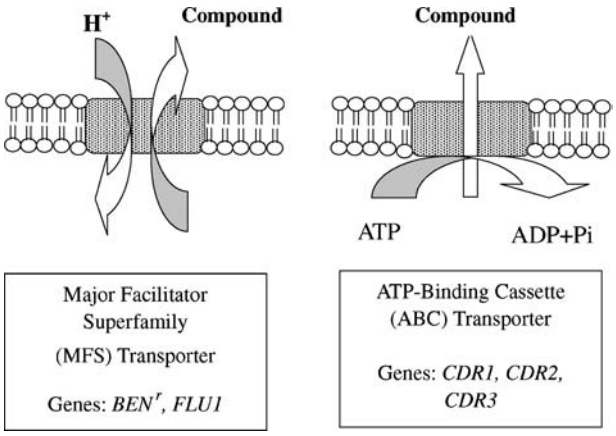

Figure 3 Schematic representation of major drug efflux systems in *Candida albicans*. At least two major classes of transporters (the MFS and the ABC transporters) are associated with multidrug resistance in *C. albicans*. Source: Niimi et al. (1997).

isolates are cross-resistant to amphotericin B because of the lack of its antifungal target, ergosterol in their membranes.

4.3 Amphotericin B

Acquired resistance to amphotericin B has been reported occasionally. As described above, the resistance to amphotericin B is often associated with alteration of membrane sterols. Dick et al. (1980) and Sokol-Anderson et al. (1988) reported that amphotericin B resistant *Candida* isolates have a marked decrease in their ergosterol content. Since the selective activity of amphotericin B against fungal cells results from its preferential association with membranes containing ergosterol (Kinsky 1961a,b; Lampen et al. 1960), it is reasonable that the lack of ergosterol leads to the lowered susceptibility to amphotericin B. On a molecular basis, Kelly et al. (1996) and Nolte et al. (1997) have shown that clinical isolates of *C. albicans* resistant to amphotericin B lack in ergosterol and accumulate 3-β-ergosta-7,22-dienol and 3-β-ergosta-8-enol, possibly due to a defect in the sterol $\Delta^{5,6}$ desaturase. Such a sterol pattern has also been found in laboratory strains of *S. cerevisiae* with a defect in the $\Delta^{5,6}$ desaturase gene *ERG3* (Kelly et al. 1995).

5 SIDE EFFECTS

In the case of topical antifungal agents for the treatment of superficial fungal infections, side effects are rarely found. Although contact dermatitis caused by the topical agents has been reported (Budimulja 1998; Dooms-Goossens et al. 1995; Goodfield 1992; Schroeff van der et al. 1992), the incidence is very low. Thus, this review covers the side effects associated with antifungal agents for the treatment of systemic fungal infections.

5.1 5-Flucytosine (5-FC)

5-Flucytosine is known to have some relatively minor gastrointestinal side effects such as nausea and diarrhea, and it also has more severe side effects including hepatotoxicity and bone marrow suppression (Vermes et al. 2000a,b). Benson and Nahata (1988) reported that gastrointestinal side effects, the most common and least harmful side effects, associated with 5-FC occur in approximately 6% of patients treated with 5-FC. In most cases of hepatotoxicity, increases in serum concentrations of transaminases and alkaline phosphatase are observed, and can usually be reversed if the dose of 5-FC is reduced and sometimes even when the dose is unchanged (Vermes et al. 2000b). The mechanism of 5-FC-associated hepatotoxicity has not been clarified yet, but it seems to be concentration-dependent, possibly avoidable if peak 5-FC concentrations are kept below 100 μg/ml (Francis and Walsh 1992; Stamm et al. 1987). The most severe side effect associated with 5-FC is bone marrow suppression. Several reports have shown that serious or life-threatening leukocytopenia, thrombocytopenia and/or pancytopenia are observed in patients treated with 5-FC (Kauffman and Frame 1977; Schlegel et al. 1970). The mechanism of 5-FC-associated bone marrow suppression is still not fully understood, but it is likely that deamination of 5-FC leads to 5-FU formation and resulting 5-FU is responsible for bone marrow suppression (Vermes et al. 2002).

5.2 Amphotericin B

Although amphotericin B is the gold standard for antifungal treatment for the most of systemic antifungal infections, side effects are commonly seen in the course of treatment. Among them is nephrotoxicity being the most serious, occurring early in the course of treatment, and usually being reversible in most patients (Sabra and Branch 1990; Sawaya et al. 1995; Miano-Mason 1997; Catald 2000). Nephrotoxicity seems related to direct amphotericin B action on the renal tubules as well as to drug-induced renal vasoconstriction (Fanos and Cataldi 2000). Although the mechanisms involved in amphotericin B-associated nephrotoxicity are not fully understood yet, a number of studies have contributed to a better understanding of the mechanisms by which amphotericin B exerts its nephrotoxic effect. Amphotericin B alters cell membrane permeability, which probably in turn affects tubular and vascular smooth muscle cell function, leading to the various tubular transport defects and vasoconstriction (Sawaya et al. 1995; Schell et al. 1989). Recent advanced technologies, however, made it possible to reduce the nephrotoxicity caused by the drug. A new approach is to complex the drug with lipids or to entrap it in liposome. Three lipid-associated formulations of amphotericin B

(amphotericin B-liposomal formulation, amphotericin B-lipid complex, and amphotericin B-colloidal dispersion) have been developed as an attempt of improving both efficacy and tolerability (Anaissie et al. 1998; Walsh et al. 1998; Walsh et al. 1999). These formulations are intravenously available and reduce the toxicity associated with amphotericin B (Meis and Verweij 2001).

5.3 Azoles

The introduction of ketoconazole, the first orally active antifungal azole, about two decades ago was a major therapeutic breakthrough, and early studies of ketoconazole suggested that the agent was remarkably free of severe side effects. In the first few thousand patients treated, about 10% of patients developed such syndromes as nausea, vomiting, headache, dizziness, constipation, drowsiness, diarrhea, nervousness, and itching (Levine 1981; Smith and Henry 1984). As more patients have been treated, significant side effects have been reported: the most serious side effects are gynecomastia, endocrine effects, and liver toxicity. Primarily in patients receiving relatively high doses of ketoconazole, gynecomastia has been found and appears to be due to inhibitory effects of ketoconazole on testosterone synthesis (DeFelice et al. 1981; Pont et al. 1982a,b; Stevens et al. 1982). In addition, evidences have been accumulated to show that the agent inhibits the synthesis of other steroids, such as corticosteroids (Pont et al. 1982b; Graybill and Craven 1983). On a molecular basis mechanism, the targets of endocrine system for overdosing with azole antifungals in laboratory animals are the adrenal cortex and the gonads because of the interaction of azoles with the cytochrome P450 enzymes involved in the glucocorticoid and sex steroid syntheses (Van Cauteren et al. 1989). Of more concern is that ketoconazole may induce live toxicity, since there have been well-documented cases of ketoconazole-induced hepatitis (Heiberg and Svejgaard 1981; Janssen and Symoens 1983; MacNair et al. 1981; Svejgaard and Ranek 1982). However, the mechanism by which ketoconazole or other azole antifungals induce hepatic dysfunction is not fully understood. Niemegeers et al. (1981) identified that ketoconazole when given to rats at a high dose is a hepatic microsomal enzyme inhibitor, and some workers (Vanden Bossche 1985; Lavrijssen et al. 1987) additionally showed that the mechanism of the effect is due to an interaction of the azole-nitrogen with the heam group of cytochrome P450 (CYP) of the microsomal enzyme. This is of importance because the presence or absence of a metabolic interaction with liver metabolism of other xenobiotics in patients can be considered as the first step in the safety evaluation of liver function. More in detail, ketoconazole and itraconazole are potent inhibitors of the major CYP isoform in humans, CYP3A4 (Back and Tjia 1991; Baldwin et al. 1995; Bourrié et al. 1996). Therefore, coadministration of these azoles with CYP3A4 substrates such as cyclosporin, tacrolimus, nifedipine, simvastatin, or terfenadine can result in clinically significant interactions, some of which can be life-threatening. Although the potency of fluconazole as a CYP3A4 inhibitor is much lower, the azole is a potent inhibitor of CYP2C9 (Kunze et al. 1996). Thus, coadministration of warfarin with fluconazole can result in clinically significant drug interaction. As a whole, the azole antifungals are all inhibitors of CYP enzymes, but their potencies are vastly different for the various isoforms (Venkatakrishnan et al. 2000).

6 FURTHER PROSPECT

Today the situation for fungal pathogens is very different, and the need for potent antifungal agents seems to become greater than ever. This need has arisen largely through the increased number of profoundly and chronically immunocompromised patients. Several new antifungal agents are currently in development, which will hopefully extend the treatment options in the near future. In azoles, there are three triazole antifungals being developed for clinical use (not yet licensed). Voriconazole has a broad spectrum of antifungal activity being active against major causative fungi of systemic fungal infections, *Candida* spp., *Aspergillus* spp., and *Cryptococcus neoformans* as well as the fungi causing endemic mycoses, *Fusarium* spp., and dematiaceous fungi (Kauffman and Zarins 1998; McGinnis et al. 1998; Nguyen and Yu 1998; Oakley et al. 1998). Thus, its main indication is expected to be treatments of invasive aspergillosis and other mold infections in immunocompromised patients. Posaconazole (SCH 56592) and ravuconazole (BMS-207147) are the two other triazoles in development. Pfaller et al. (1998) compared *in vitro* activity of these triazoles with that of voriconazole against 1,300 bloodstream isolates of *Candida* spp., resulting in high activity of all compounds tested against the *Candida* spp. including *C. glabrata*. These two triazoles proved also highly active against *Aspergillus* spp. (Manavathu et al. 2000; Moore et al. 2000). As for the treatment of superficial fungal infections, a topical imidazole compound, NND-502, is in development in Japan. NND-502 exerts extremely potent *in vitro* and *in vivo* antidermatophyte activities so that it is expected to be highly effective in the short-term treatment of dermatomycoses in humans (Niwano et al. 1998). Recently, as a major advance in antifungal chemotherapy, a new class of antifungal drugs, the echinocandins, has been developed. The echinocandins work by inhibition of $\beta(1-3)$-glucan synthase, resulting in prevented incorporation of glucans into the fungal cell wall (Bartizal et al. 1997; Kurtz and Rex 2001). One preparation in clinical studies is caspofungin (MK-0991), which has been shown to exert promising activity against the important fungal pathogens including *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae*, and *Aspergillus* spp. (Bartizal et al. 1997; Barchiesi et al. 1999; Pfaller et al. 1999). In addition, it enhances the activity of amphotericin B against *C. neoformans* and *Aspergillus* spp., suggesting that this may be useful in combination with this established antifungal

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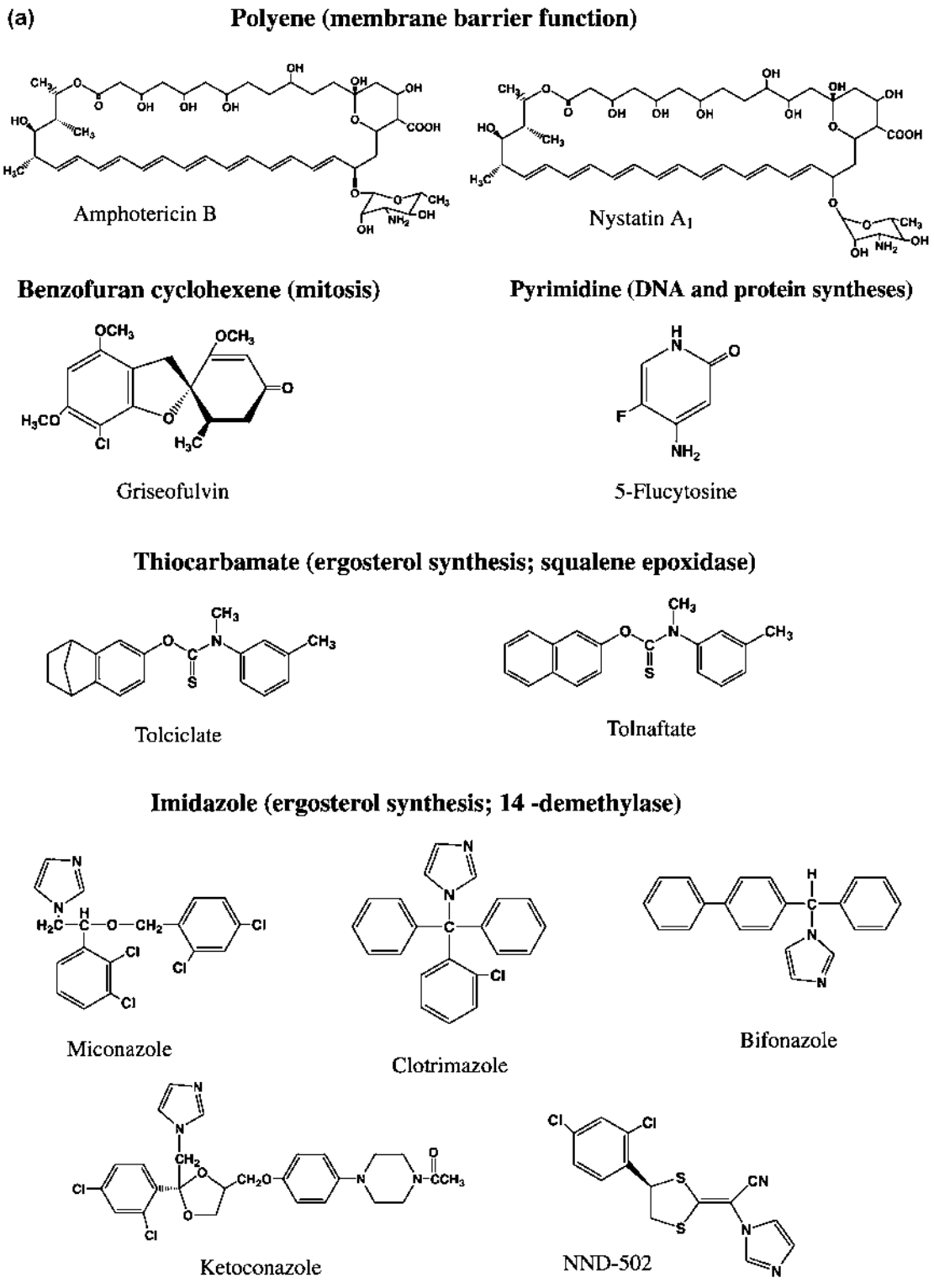

Figure 4 (a) Chemical structures and antifungal targets of important classes (polyene, benzofuran cyclohexene, pyrimidine, thiocarbamate, and imidazole) of antifungal agents. Antifungal targets are shown in parentheses. (b) Chemical structures and antifungal targets of important classes (triazole, allylamine, benzylamine, morpholine, and echinocandin) of antifungal agents. Antifungal targets are shown in parentheses.

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agent (Franzot and Casadevall 1997; Arikan et al. 2002). The most recent echinocandin entering clinical trials is FK463. Although published data on it are limited, the agent seems to be similar to caspofungin. FK463 displays substantial antifungal activity against both *Candida* spp. and *Aspergillus* spp. (Tawara et al. 2000), and proved efficacious in several animal models of systemic fungal infections (Ikeda et al. 2000; Maesaki et al. 2000; Matsumoto et al. 2000). FK463, like other echinocandins, have also proved active against azole-resistant strains of *Candida albicans* expressing *CDR1* or *CaMDR* (Maesaki et al. 2000). Since these echinocandins are generally well tolerated and free from significant interaction with cytochrome P450 (Groll and Walsh 2001), they appear as promising candidates for the next generation of antifungal agents.

7 CONCLUSIONS

Efforts have been made to discover desirable antifungal agents with selectivity to pathogenic fungi since the beginning of the twentieth century, and such potent antifungal agents as undecylenic acid, griseofulvin, 5-FC, polyene derivatives (nystatin and amphotericin B), thiocarbamate

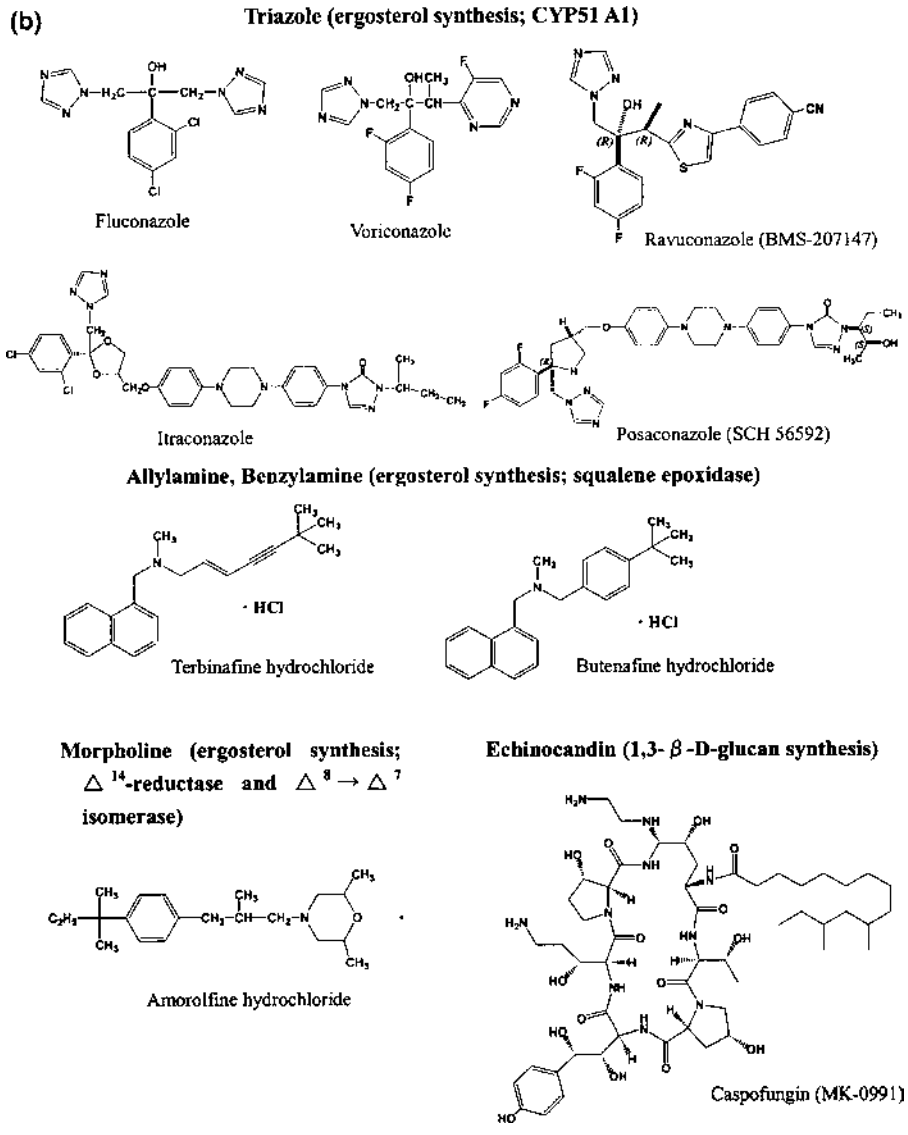

Figure 4 (*continued*)

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derivatives (tolnaftate, tolciclate, and liranaftate), imidazole derivatives (clotrimazole, miconazole, ketoconazole, and bifonazole), ciclopirox olamine, triazole derivatives (fluconazole and itraconazole), amorolfine, allylamine derivatives (naftifine and terbinafine), and butenafine were successfully discovered and developed for the treatment of superficial and/or systemic fungal infections. Chemical structures and antifungal targets of important classes of antifungal agents are shown in Figure 4a and b. Among them, undecylenic acid, thiocarbamate derivatives, imidazole derivatives, ciclopirox olamine, amorolfine, allylamine derivatives, and butenafine are topically used for treating superficial fungal infections. In the current management of tinea infections, for example, generally more than 80% of patients are cured by the topical treatment with imidazoles, allylamines, butenafine, or amorolfine for 2 to 4 weeks. In addition, most cases of tinea ungium and other fungal infections of the nails (onychomycosis) can be treated effectively with oral itraconazole or oral terbinafine. Unlike superficial fungal infections, systemic fungal infections are generally life-threatening to immunocompromised people, and the medical challenge of management for systemic fungal infections are partially conducted by the prophylactic, empiric, and therapeutic use of antifungal drugs such as amphotericin B, 5-FC and a series of azoles. Amphotericin B is a broad-spectrum and potent antifungal agent, but the clinical use of the agent is sometimes limited because of adverse reactions such as renal toxicity, hypokalemia and anemia. 5-FC has a relatively narrow antifungal spectrum, and especially exerts potent activity against *Candida* spp. and *Cryptococcus neoformans*. But it should not be administered as a single drug because of the frequent development of acquired drug resistance. The introduction of the triazole antifungal agents, fluconazole and itraconazole, has challenged amphotericin B as the gold standard for the treatment and prevention of systemic fungal infections. Fluconazole, however, is comparably less active against *Aspergillus* spp. than yeast-type fungi, and oral itraconazole is poorly absorbed in humans because of its high lipophilicity and low water solubility. Furthermore, resistance to these triazole agents is growing with their increasing use for a relatively long-term therapy. The mechanisms by which yeastlike fungi such as *C. albicans* acquire resistance to azole antifungals are as follows: (a) cells fail to accumulate these compounds, (b) the affinity of CYP51A1 to these compounds is altered, (c) the content of CYP51A1 increases in cells, (d) the sterol $\Delta^{5,6}$ desaturase is inactivated, and (e) a combination of these mechanisms. To overcome these problems, reformulations of existing antifungal agents are developed, and the search for new treatment options for life-threatening systemic fungal infections continues. Three lipid-associated formulations of amphotericin B are developed in attempts to improve both efficacy and tolerability. Two new formulations of itraconazole are also developed by using hydroxypropyl-β-cyclodextrin as a solubilising excipient to improve oral bioavailability and to be available for intravenous use. As new treatment options, new triazole derivatives such as voriconazole, posaconazole (SCH 56592) and ravuconazole (BMS-207147) are in development and their efficacy is assessed in clinical trials. In addition, the members of echinocandin class working as a β(1–3)-glucan synthase inhibitor are in development, and caspofungin (MK-0991) and FK463, for example, are now entering the clinical trials. These compounds are expected to minimize mechanism-based toxicity and to act on fungi with intrinsic or acquired resistance to existing drugs because of the fungal-specific mode of action.

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Antitumor and Immunomodulatory Compounds from Fungi

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1 INTRODUCTION

Cancer is one of the major fatal diseases worldwide, and affects the people of all ages. Not all cancers can be detected at an early stage; lung cancer is an example of a cancer that is difficult to detect early and this explains the poor prognosis. Currently available cancer treatments include surgery to remove a cancer that has not undergone metastasis followed by radiotherapy or chemotherapy to eradicate residual cancer cells, radiotherapy for treatment of certain cancers like nasopharyngeal cancer and cancers that have progressed beyond the early stage, and chemotherapy for cancers that have metastasized. Immunotherapy is also a possible method of treatment that can be used to boost the function of the immune system in the fight against cancer cells. Radiotherapy and chemotherapy suffer from the drawback that normal cells are also affected, resulting in loss of hair and appetite and deterioration in hematologic parameters. In view of the undesirable side effects of radiotherapy and chemotherapy, attention has been drawn to alternative and complementary forms of treatment. Some of these compounds, such as polysaccharopeptide PSP and the protein-bound polysaccharide from *Coriolus versicolor* and *Ganoderma* polysaccharide, are commercially available in some countries, especially those in the orient. Clinical trials have been conducted with these compounds and it has been found that in general, there is an improvement in the immune status of cancer patients undergoing radiotherapy or chemotherapy. Tumor regression has occurred and survival rate has been prolonged in some of these patients. However, the possibility of exploiting other fungal compounds with similar activities in cancer therapy or immunomodulation has not been widely explored, nor has the possible use of such fungal products as prophylactics. It is worth pointing out that the antibiotic penicillin and the cholesterol-lowering drug lovastatin are examples of fungal products successfully developed into therapeutic agents. They mark only the beginning of the use of fungal products in the field of medicine and more may well follow. This chapter focuses on the variety of fungal compounds exhibiting immunomodulation and antitumor activities.

2 PROTEINS AND PEPTIDES WITH ANTITUMOR AND IMMUNOMODULATORY ACTIVITIES

2.1 Ubiquitin-Like Peptides

Ubiquitin, as its name implies, is found in a variety of tissues and organisms. However, only a few ubiquitinlike peptides have been reported from fungi. A peptide with a molecular mass of 8 kDa, and an ubiquitinlike N-terminal sequence, has been purified from the fruiting bodies of the mosaic puffball mushroom *Calvatia caelata*, (Lam et al. 2001). Ubiquitinlike peptides have also been found in *Pleurotus ostreatus* (Wang and Ng 2000) and *Pleurotus sajor-caju* cv hsiu tseng (Ng et al. 2002). Ubiquitin plays a role in the degradation of important regulatory proteins including many cell cycle regulatory proteins, p53 tumor suppressor, the transcriptional regulator NF-KB and its inhibitor, many transcription factors, and the mos proto-oncogene. The ubiquitin-mediated pathway is essential to the control of cell cycle progression, signal transcriptional regulation, receptor down-regulation, endocytosis, immune response, development and apoptosis. Abnormalities in ubiquitin-mediated processes have been implicated in the etiology of pathological conditions including malignant transformation (Hershko 1998). The physiological role of fungal ubiquitinlike peptides and proteins may be related to these functions.

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The ubiquitin-like peptide from *Calvatia caelata* (CULP) has shown an anti-proliferative action on human breast cancer (MDA-MB-231) cells with an IC_{50} of 100 mM. The peptide also exerts an antimitogenic action on mouse splenocytes with an IC_{50} of about 100 n*M*. The antimitogenic and anti-proliferative activities of CULP may be due to its ability to inhibit translation. The inhibitory effect of fungal ubiquitin-like peptides on cell-free translation is in line with the role of the ubiquitin–proteasome pathway in degradation of short-lived and regulatory proteins involved in various cellular processes.

2.2 Ribosome Inactivating Proteins

Ribosome inactivating proteins are well known for their ability to inhibit translation in a cell-free rabbit reticulocyte lysate system and for their N-glycosidase activity (Lam et al. 1996). The ribosome inactivating protein isolated from fruiting bodies of *Lyophyllum shimeji* inhibits incorporation of methyl [^{3}H] thymidine into mouse splenocytes, in line with the anti-mitogenic activity shown by angiosperm ribosome inactivating proteins (Lam and Ng 2001). α-Sarcin is a cytotoxic polypeptide produced by *Aspergillus giganteus* that shows antitumor activity (Martinez del Pozo et al. 1988). An intratumoral injection of α-sarcin (0.4 mg/tumor) into an heterotransplanted human pulmonary adenocarcinoma grown in naked mice produces four ultrastructural changes in the tumor cells: (a) mitochondrial swelling, (b) cell necrosis with partial phagocytic removal of necrotic cells, (c) thickening of interlobular connective tissue, and (d) hyperplasia of goblet-cell-like clear cells. It has been suggested that selective membrane permeabilization is required before α-sarcin can interact with different intracellular structures (Mohamed-Ali et al. 1991).

2.3 Lectins

The antitumor and immunomodulatory activities of lectins from nonfungal sources have been extensively reported. Fungal lectins also have similar attributes and the anti-proliferative and antitumor activities of a number of these have been demonstrated. *Agaricus bisporus* lectin inhibits the incorporation of ^{3}H-thymidine into human colon cancer cell lines HT29 and Caco-2, breast cancer cell line MCF-7 and rat mammary fibroblasts Rama-27, the rates of inhibition being 87, 16, 50, and 55%, respectively, at a lectin concentration of 25 μg/ml. At a concentration of 10 μg/ml the lectin completely cancels out the stimulatory effect of epidermal growth factor (100 pg/ml) and drastically diminishes the enhancement of insulin (50 ug/ml) on [^{3}H] thymidine incorporation (Yu et al. 1993). *Boletus satanas* lectin has been shown to have a potent mitogenic activity on human peripheral blood lymphocytes, and also to stimulate the release of interleukin-1α, interleukin-2, and tumor necrosis factor-α from mononuclear cell cultures (Licastro et al. 1993). An immunomodulatory protein from *Flammulina velutipes*, designated FIP-fve, exerts a stimulatory effect on human peripheral blood lymphocytes, inhibits systemic anaphylaxis reactions and local swelling of mouse footpads, and augments transcriptional expression of interleukin 2 and interferon-γ (Ko et al. 1995). An immunomodulatory protein from *Ganoderma lucidum* has been reported to exert a mitogenic activity on mouse splenocytes and human peripheral lymphocytes, and to suppress anaphylaxis induced by bovine serum albumin in CFW mice (Haak et al. 1993). *Grifola frondosa* lectin is cytotoxic against HeLa cells, and a minimum concentration of 25 μg/ml results in the death of all of the cells. This toxicity disappears after preincubating the lectin with the haptenic sugar *N*-acetylgalactosamine (Kawagishi et al. 1990). A heterodimeric melibiose-binding lectin from fruiting bodies of the oyster mushroom *Pleurotus ostreatus* has been shown to be a potent inhibitor of sarcoma S-180 and hepatoma H-22 in mice and lectin treatment (1.5 mg lectin/kg body weight/day for 20 days) leads to a drastic reduction in tumor weight. Lectin treatment results in 88% and 75% inhibition of sarcoma and hepatoma growth 21 days after inoculation of tumor cells. There is 58% and 107% increase in the survival time of sarcoma-bearing mice that have been inoculated with 5×10^6 and 2.5×10^5 tumor cells respectively, and 22% and 38% increases in the survival times for hepatoma-bearing mice that have been inoculated with 5×10^6 and 2.5×10^5 tumor cells, respectively (Wang et al. 2000).

A novel lectin from the fruiting bodies of the straw mushroom *Volvariella volvacea* has shown a stronger immunomodulatory effect than concanavalin A (She et al. 1998). *Tricholoma mongolicum* produces two lectins, TML-1 and TML-2, and of these TML-1 is the more potent in anti-proliferative activity against mouse monocyte-macrophage PU5-1.8 cells whereas both lectins are equally effective in their anti-proliferative activity against mouse mastocytoma P815 cells. Both lectins inhibit tumor growth and prolong the life-span of tumor-bearing BALB/c mice, which have been intraperitoneally inoculated with sarcoma S180 cells. However, there is no inhibitory effect on the growth of S180 cells *in vitro*, which suggests that the lectins are immunomodulatory substances rather than substances exerting direct cytotoxicity. Peritoneal macrophages in mice treated with *Tricholoma mongolicum* lectin TML-1 or TML-2 show, on lipopolysaccharide stimulation, an enhanced production of nitrite and tumour necrosis factor. TML-2 inhibits the growth of P815 mastocytoma cells better than TML-1 by stimulating peritoneal macrophages to produce more macrophage activating factors including interferon-γ and some other cytokines. However, the lectins do not stimulate T cells from normal or sarcoma-bearing mice (Wang et al. 1995b; 1996a; 1997).

2.4 Lysine Oxidase

Lysine oxidase is an antitumor enzyme that has been reported from an isolate of *Trichoderma* sp. and this has been

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shown to produce an antiinvasive effect *in vitro* and an anti-metastatic activity *in vivo*. Pre-treatment of tumor cells (MM1 clone cells) with 2.5 mU/ml of lysine oxidase has resulted in a 1.9-fold decrease in cell growth and a 1.6-fold decline in invasive capacity. Treatment of mice with lysine oxidase (50 U/kg, i.v.) has led to a reduction in the extent and number of Lewis lung metastases, as well lengthening their life span (Khaduev et al. 1991).

2.5 Collagen Binding Protein

A 41 K collagen binding protein, HM41, has been isolated from *Hypsizigus marmoreus*. This protein has a cell adhesion-promoting activity for murine Lewis lung carcinoma LL2 cells and human fibrosarcoma HT1080 cells, and an affinity for type IV collagen. It interacts with both animal extracellular matrix protein type IV collagen and with animal tumor cells. However, Western blotting with anti-HM41 antibodies has shown that HM41 is not related to HM23, although the latter also exhibits an affinity for type IV collagen, Cyanogen bromide fragments of HM41 show no close homology with any currently known proteins (Shoji et al. 2000).

3 POLYSACCHARIDES AND PROTEIN-BOUND POLYSACCHARIDES

There are many reports of angiosperm polysaccharides having anti-tumour and immunomodulatory activites. A vast number of fungal polysaccharides and protein-bound polysaccharides demonstrate antitumor activity and these include polysaccharides from *Agaricus blazei* (Mizuno et al. 1999), *Amanita muscaria* (Kiho et al. 1994), *Armillariella tabescens* (Kiho et al. 1992), *Auricularia* species (Ukai et al. 1983), *Candida albicans* (Ausiello et al. 1987), *Dictyophora indusiata* (Ukai et al. 1983), *Flammulina velutipes* (Otagiri et al. 1983), *Lentinus edodes* (Chihara et al. 1970), *Omphalia lapidescens* (Ohno et al. 1992), *Pleurotus ostreatus* (Yoshioka et al. 1985), *Pleurotus citrinopileatus* (Zhang et al. 1994), *Poria cocos* (Kanayama et al. 1983), and *Saccharomyces cerevisiae* (Suzuki et al. 1969). Protein-bound polysaccharides with antitumor activity include those from *Cordyceps ophioglossoides* (Ohmori et al. 1988), *Coriolus versicolor* i.e., PSK (Hirose et al. 1985) and PSP (Wang et al. 1996b; Wan et al. 1999), *Ganoderma lucidum* (Miyazaki and Nishijima 1981), *Ganoderma tsugae* (Zhang et al. 1994), *Hehenbuehelia serotina* (Ma et al. 1991), *Hericium erinaceum* (Mizuno et al. 1992), *Sparassis crispa* (Ohno et al. 2000), *Tricholoma lobayense* (Mizuno et al. 1995; Wang et al. 1995a), *Tricholoma mongolicum* (Wang et al. 1996c), and *Volvariella volvacea* (Kishida et al. 1989).

Immunostimulation has been shown by polysaccharides from *Agaricus blazei* (Fujimiya et al. 1999), *Agrocybe cylindracea*, and *Amanita muscaria* (Yoshida et al. 1996), *Armellaria tabescens* (Kiho et al. 1992), *Candida albicans* (Domer et al. 1988), *Flammulina velutipes* (Leung et al. 1997; Ohkuma et al. 1993), *Grifola frondosa* (Ishibashi et al. 2001), *Lentinus edodes* (Tani et al. 1993), *Phellinus linteus* (Song et al. 1995), *Sarcodon aspratus* (Mizuno et al. 2000), *Schizophyllum commune* (Sakagami et al. 1988) and *Sclerotinia sclerotiorum* (Sakurai et al. 1995). Protein-bound polysaccharides from *Coriolus versicolor* i.e., PSK (Sakagami et al. 1991) and PSP (Lee et al. 1999; Liu et al. 1993), *Tricholoma lobayense* and *Tricholoma mongolicum* (Ng et al. 1999; Wang et al. 1995b; 1996c) also have immunostimulating effects.

Polysaccharides and/or polysaccharide–protein complexes from the following fungi: *Agaricus blazei* (Mizuno et al. 1999). *Amanita muscaria* (Kiho et al. 1994), *Armillariella tabescens* (Kiho et al. 1992), *Auricularia species* (Ukai et al. 1983), *Coriolus versicolor* (Wang et al. 1996b), *Dictyophora indusiata* (Ukai et al. 1983), *Flammulina velutipes* (Otagiri et al. 1983), *Ganoderma tsugae* (Zhang et al. 1994), *Hohenbuehelia serotina* (Ma et al. 1991), *Omphalia lapidescens* (Ohno et al. 1992), *Pleurotus citrinopileatus* (Zhang et al. 1994), *Sparassis crispa* (Ohno et al. 2000), *Tricholoma lobayense* (Wang et al. 1995a) and *Tricholoma mongolicum* (Wang et al. 1996c) have been reported for antitumor effects on mice bearing sarcoma 180 cells. These polysaccharides and/or polysaccharide-protein complexes can also have an antitumor activity on other tumors. The *A. blazei* preparation is effective in mice with *Ehrlich ascites* carcinoma, shionogi carcinoma 42 and meth A *fibrosarcoma* (Mizuno et al. 1999) and the *Flammulina velutipes* protein-bound polysaccharide has prolonged the life of mice challenged with leukemia cells (Otagiri et al. 1983). The protein-bound polysaccharide PSK isolated from *Coriolus versicolor,* and the polysaccharide-peptide PSP from *Coriolus versicolor*, have also shown effects in cancer patients (Kobayashi et al. 1995; Yang 1999).

The immunoenhancing activity of fungal polysaccharides and polysaccharide-peptides has been demonstrated in a number of ways. Polysaccharides and/or polysaccharide–peptides from *Agrocybe cylindracea* and *Amanita muscaria* (Yoshida et al. 1996), *Armillariella tabescens* (Kiho et al. 1992), *Coriolus versicolor* (Liu et al. 1993), *Grifola frondosa* (Ishibashi et al. 2001), *Lentinus edodes* (Tani et al. 1993), *Sarcodon aspratus* (Mizuno et al. 2000), *Schizophyllum commune* (Sakagami et al. 1988), *Tricholoma lobayense* and *Tricholoma mongolicum* (Wang et al. 1995a; Wang et al. 1996c) stimulate macrophages, whereas lymphocyte stimulation has been reported as a response to polysaccharides and/or polysaccharide–peptides from *Armillaria tabescens* (Kiho et al. 1992), *Coriolus versicolor* (Wang et al. 1996b), *Flammulina velutipes* (Otagiri et al. 1983), *Lentinus edodes* (Tani et al. 1993), and *Phellinus linteus* (Song et al. 1995).

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4 SMALL MOLECULES WITH ANTITUMOR ACTIVITY

4.1 Indolecarbazole Alkaloids

4′*N*-methyl-5′-hydroxystaurosporine and 5′-hydroxystaurosporine are two new indolecarbazole alkaloids with cytotoxic activity against tumor cell lines. Both compounds were isolated from the culture broth of a marine isolate of *Micromonospora* sp. (Hernandez et al. 2000).

4.2 Antitumor Antibiotics

Pyrrolosporin A has been isolated from a strain of *Micromonspora* sp. and has been reported as an antitumor antibiotic with weak activity against Gram-negative bacteria and stronger activity against Gram-positive bacteria. Polysporin A is reported to prolong the life of mice inoculated with P388 leukemia cells (Lam et al. 1996). Myrocin C is a diterpene antitumor antibiotic isolated from the culture filtrate of an isolate of *Myrothecium verrucaria*. It has been shown to prolong the life of mice with Ehrlich ascites carcinoma and also has activity against Gram-positive bacteria, fungi, and yeasts (Nakagawa et al. 1989). Saintopin is a further antitumor antibiotic from *Paecilomyces* species with topoisomerase II dependent DNA cleavage activity (Yamashita et al. 1990). Lovastatin is a fungal antibiotic as well as an inhibitor of hydroxymethylglutaryl CoA reductase, the key enzyme in the cholesterol biosynthetic pathway. It can reduce tumor formation in mice inoculated with F3II sarcomatoid mammary carcinoma cells. Lovastatin also inhibits metastatic dissemination of established mammary tumors to the lungs. Lovastatin is reported to inhibit tumor cell attachment and migration *in vitro* (Alonso et al. 1998).

4.3 Antifungal Drugs

All azole drugs except fluconazole are immunosuppressive. Azole drugs do not activate polymorphonuclear leukocytes, nor do they induce macrophage production of tumor necrosis factor-α. The polyene antibiotic amphotericin B, on the other hand, exerts an immunostimulatory action. It induces host resistance to *Pseudomonas aeruginosa* infection in mice, activates polymorphonuclear leukocytes, and induces *in vitro* production of tumor necrosis factor-α by macrophages. It primes macrophages *in vitro* and *in vivo* to produce large quantities of tumor necrosis factor-α following secondary stimulation by bacterial lipopolysaccharide OK 432, a streptococcal preparation used for antitumor immunotherapy. It also enhances the activity of viable or heat-killed *Candida albicans* cells to induce macrophage production of tumor necrosis factor *in vitro* (Yamaguchi et al. 1993).

4.4 Fungal Growth Product

6MFA has been described as a growth product of *Aspergillus ochraceus* in stationary liquid culture. Treatment of mice bearing Ehrlich ascites tumor with 6MFA has led to a reduction in body weight increase due to ascites, a reduction in the mortality rate, and an increase in survival time. 6MFA appears to have both interferon-inducing and antiviral activites under both *in vitro* and *in vivo* conditions (Mall et al. 1991).

4.5 Fungal Sesquiterpenes

The fungal sesquiterpene illudin S can be combined with excess paraformaldehyde in dilute sulfuric acid to give hydroxymethylacylfulvene. This compound is more toxic than its precursor acylfulvene, but less toxic than illudin S to HL 60 cells (McMorris et al. 1996).

4.6 Taxol

Taxol production has been reported from *Pestalotiopsis microspora*, an endophytic fungus from the Himalyan yew *Taxus wallachiana* (Strobel et al. 1996). The isolation and characterization of the diterpenoid taxol were first reported by Wani et al. (1971) from the bark of *Taxus brevifolia* but the low yield and its poor solubility in water restricted its development as an anticancer drug. Taxol has been shown to be effective in newly developed assays including mammary, lung and colon xenografts and the B16 mouse melanoma assay, where it exhibits antitumor activity. It has been found that taxol promotes the assembly of tubulin into stable microtubules and thus presumably prevents cell death. A formulation of taxol was eventually developed using cremophor EL, a polyethoxylated castor oil, and absolute ethanol, and this is diluted with 5% dextrose in water or normal saline before use (National Cancer Institute 1991; Kingston 1993). Clinical trials have been conducted on taxol and LD_{50} values determined (Holmes et al. 1991; McGuire et al. 1989).

4.7 Fungal Toxins

Fungal toxins have recently has been reviewed by Bondy and Pestka (2000); also see chapters in this book). Aflatoxin acts mainly on cell-mediated immunity and phagocytic cell function. Its immunotoxicity can be ameliorated by amending or supplementing the diet. Exposure of mice to the levels of patulin encountered in feeds and foods does not result in immunotoxicity. Trichothecenes can both stimulate and suppress immune function. MT81, a mycotoxin from *Penicillium nigricans*, has been shown to prolong the life of mice with Ehrlich ascites carcinoma by 78%, and mice with

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sarcoma 180 by over 100%. In these cases, there is a reduction in the tumor volume and viable tumor cell count. The tumor cells in the treated animals are characterized by a reduced mitotic activity and the presence of membrane blebbing and cytoplasmic vacuoles (Gupta et al. 1997). The treated animals have an improved hematological profile.

Three mycotoxins, designated destruxin A, B, and E, have been isolated from *Metarhizium anisopliae*. Destruxin E has a strong anti-proliferative activity against P388 leukemic cells, and causes the cells to accumulate in the GO/1 phase. All three destruxins can reduce the number of cells in the G2 phase although destruxins A and B do not affect the number of cells in the GO/1 phase (Odier et al. 1992).

The effects of exposure of the immune system of mice and rats to ochratoxins in the prenatal and perinatal periods have been reviewed, and fumonisin-induced immunotoxicity is under intensive investigation. Ochratoxin causes a dose-dependent immunomodulation in weaner pigs even when present in subtoxic amounts. Ochratoxin causes an increase in the counts of leukocytes, neutrophils, eosinophils, and apoptotic phagocytes but a decreased lymphocyte count. Production of reactive oxygen radicals in whole blood is markedly elevated, accompanied by reduced phagocytosis and diminished expression of a swine-specific surface marker on lymphocytes. Lung clearance, the degree of severity of experimental pneumonia, and cutaneous hypersensitization are also affected (Muller and Kielstein 1999).

Immunosuppression has been demonstrated for gliotoxin *in vitro*, but the association between immunosuppression and the presence of gliotoxin in infected tissues *in vivo* has not been determined. Gliotoxin reduces the immune response of murine macrophages *in vitro*. It modulates the immunogenicity of murine bone marrow cells, and can delay the onset of graft-versus-host disease in fully allogeneic, bone marrow chimeras. Gliotoxin, at 10 μg/ml, can reduce the responsiveness of human pancreatic, splenic, hepatic, and bone marrow cells in mixed lymphocyte cultures. The same gliotoxin cocentration reduces insulin secretion from pancreatic explants, but only during the first day of organ culture. Three months after tissue implantation beneath the renal capsule in nude mice, gliotoxin-treated pancreatic explants are lighter and contain less insulin than the untreated controls. This difference is indiscernible from three weeks after transplantation. It has been hypothesized that the immunomodulating effect of gliotoxin, at a concentration of less than 10 μg/ml, may be beneficial in treating allografted human fetal pancreas before transplantation, as it has for adult bone marrow cells in mice (Tuch et al. 1988). Gliotoxin noncompetitively inhibits the chymotrypsinlike activity of the 20S proteasome *in vitro*. Reduction of gliotoxin by dithiothreitol reverses this proteasome inhibition. In intact cells, gliotoxin induces the transcription factor NF-kappa B by suppressing proteasome-mediated degradation of the NF-kappa B inhibitor lkappa Balpha. Gliotoxin also suppresses antigen processing and induces macrophagocytic apoptosis (Kroll et al. 1999).

T-2 toxin is a trichothecene mycotoxin produced by several *Fusarium* species. A stresslike response occurrs following exposure to T-2 toxin, and blood levels of corticosterone and endotoxin and hypothalamic level of norepinephrine are all elevated. This results in forestomach ulceration, together with lymphocyte infiltration, epithelial proliferation, and hyperkeratinization and the spleen becomes enlarged with a proliferative red pulp and hepatomegaly occurs but no histological changes are detectable. Degeneration of thymus cells is attributable to a heightened corticosterone level, and there is a reduction in the T-dependent antibody response (Taylor et al. 1989).

5 CONCLUSION

Fungi produce a variety of structurally diverse, high- and low-molecular-weight molecules, that stimulate the immune system, inhibit proliferation of tumor cells, and retard tumor growth (Tables 1 and 2). This makes fungi potentially valuable sources for the development of therapeutic agents against malignant diseases. The compounds with antitumor and immunomodulatory activities include ubiquitin-like peptides, ribosome inactivating proteins, lectins, polysaccharides and polysaccharopeptides, antibiotics, mycotoxins, and antifungal drugs. It is anticipated that significantly more antitumor and immunomodulatory compounds of fungal origin will be identified in the future.

Fungal ubiquitin-like peptides, ribosome inactivating proteins, lectins, and lysine oxidase are proteins with antitumor and/or immunomodulatory activities and of all these fungal proteins, lectins are the most well known. The literature about fungal ubiquitinlike peptides, ribosome inactivating proteins and lysine oxidase is limited, as they

Table 1 N-terminal sequences of some fungal proteinaceous compounds with antitumor and/or immunomodulatory activity

Calvatia caelata ubiquitin-like peptide	MQIFVKTLTGKTITLEVEESDDIDNNKAKI
Lyophyllum shimeiji ribosome inactivating protein	ITFQGASPARQTVITNAITRARADVRAAVSA
Pleurotus ostreatus lectin (40 K subunit)	ATAKIKATPAQPQQFQPAALNAAK
Pleurotus ostreatus lectin (41 K subunit)	ACATAKCTTATPQQPGCAPAALNAAK
Volvariella volvacea lectin	PSNGNQYLIAQAYNLQKVNFDYTPQWQRGN

Data are from Lam et al. (2001); Lam and Ng (2001); She et al. (1998); Wang et al. (2000).

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Table 2 Fungal products with antitumor and/or immunomodulatory activities

Ubiquitin-like peptides	Polysaccharides and protein-bound polysaccharides
Ribosome inactivating proteins	Antitumor antibiotics
	Antifungal drugs
Lectins	Taxol
Lysine oxidase	Alkaloids
Collagen binding protein	Mycotoxins

have only recently been described. In contrast, mushroom lectins have been known for at least three decades. Interest in mushroom lectins stems from research on plant and animal lectins that first showed many of their exciting biological activities. In spite of the proteinaceous nature of lectins, some plant lectins have been demonstrated to resist proteolytic digestion in the gastrointestinal tract. The fungal ubiquitinlike peptides are more stable than proteins due to their peptide nature. Mushroom lectins with different carbohydrate-binding specificities exhibit both antitumor and immunomodulatory activities, similar to the situation with plant and animal lectins. Both concanavalin A which binds glucose and mannose, and wheat germ lectin which binds *N*-acetylglucosamine and sialic acid, exert an anti-proliferative activity on tumor cells *in vitro*.

It is noteworthy that lectin from the straw mushroom exerts a more potent immunostimulating action than concanavalin A (She et al. 1998), when concanavalin A is already renowned as a strong immunostimulant. Many of the mushroom lectins that have antitumor and immumomodulatory effects are derived from common edible mushrooms including the button mushroom *Agaricus bisporus*, the winter mushroom *Flammulina velutipes*, the medicinal mushroom *Ganoderma lucidum*, the oyster mushroom *Pleurotus ostreats* and the straw mushroom *Volvariella volvacea*. Good yields of these lectins can be obtained from these mushrooms using simple chromatographic procedures. Further in-depth studies on the mechanisms of the immunomodulatory and antitumor actions of these mushroom lectins are required and the use of state-of-the-art methodology such as flow cytometry should be encouraged in order to obtain comparable data to that already known for plant lectins.

Fungal polysaccharides, protein-bound polysaccharides, and polysaccharopeptides constitute another group of compounds with activity on tumor cells and immune cells. Many of these compounds are also produced by common edible mushrooms. In addition to proteinaceous compounds, fungi also produce small molecules with antitumor and/or immunomodulatory activities. It is interesting to note that some fungal toxins have an immunosuppressive action as well as other toxic effects. However, other mycotoxins such as the destruxins (Odier et al. 1992) and MT81 from *Penicillium nigricans* (Gupta et al. 1997) display an antiproliferative action. It remains to be seen whether destruxins and MT81 can be developed into drugs for cancer therapy. The same applies to the antitumor antibiotics produced by fungi.

The antitumor plant compound taxol is also produced by the endophytic fungus *Pestalotiopsis microspora* from the Himalyan yew *Taxus wallachiana* (Strobel et al. 1996). The ups and downs of research on taxol illustrate the perseverance necessary to obtain a usable product. Lysine oxidase from a *Trichoderma* species has been shown to exhibit antimetastatic activity (Khaduev et al. 1991), and further studies are required to investigate if this can be developed into a drug for therapeutic purposes. Examination of other *Trichoderma* species and other fungal species will determine if the antitumor enzyme is restricted in its occurrence and/or yield. The finding that the fungal product lovastatin, an antibiotic as well as a drug for treating hypercholesterolemia, manifests antitumor and antimetastatic activities (Alonso et al. 1998), is interesting. It remains to be seen whether lovastatin can be applied in cancer therapy. Overall, fungi produce a diverse range of compounds with antitumor and immunomodulatory activities. Some of these, such as the protein-bound polysaccharide PSK and the polysaccharopeptide PSP from *Coriolus versicolor* and *Ganoderma lucidum* polysaccharide and taxol are well-known marketable compounds. It is hoped that with time more fungal compounds can be developed into therapeutic agents.

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Clinical and Laboratory Diagnosis of Fungal Infections

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1 INTRODUCTION

The last 20 years have seen unprecedented changes in the pattern of fungal infection in humans. These infections have assumed a much greater importance because of their increasing incidence in patients with the acquired immunodeficiency syndrome (AIDS), in transplant recipients, in cancer patients, and in other groups of debilitated or immunocompromised individuals. New pathogens have emerged, while others that once were common have almost been eradicated. Major changes in medical practice, increasing international travel, and misuse of antimicrobiol agents, are among the factors that have contributed to this changing pattern of fungal infection. New drugs have been developed, resulting in a choice of treatment depending, to some extent, on the infection and the underlying condition of the patient.

1.1 The Nature of Fungi

Living organisms are now divided up among no fewer than five Kingdoms, one of which is the Kingdom Fungi. This Kingdom consists of a diverse group of eukaryotic organisms, found throughout nature, which absorb their nourishment from living or dead organic matter.

Classification and identification of fungi is based on their appearance, rather than on the nutritional and biochemical differences that are of such importance in bacterial classification. In moulds, the vegetative stage consists of branching filaments or hyphae that together form the mycelium. While the hyphae of the more primitive moulds remain aseptate (without cross walls), those of the more advanced groups are septate, with more or less frequent cross-walls. The individual reproductive bodies of fungi, or spores, consist of a single cell or several cells contained within a rigid wall. During their evolution, most fungi have relied on a combination of sexual and asexual reproductive mechanisms to assist their survival. Their sexual spores and the structures that develop around them form the main basis for fungal classification. In some fungi, however, the asexual stage has proved so successful as a means of rapid dispersal to new habitats that the sexual stage has diminished or even disappeared. In these fungi, the shape of the asexual spores and the arrangement of the spore-bearing structures are of major importance in identification.

Yeasts are unicellular fungi consisting of separate round, oval, or elongated cells that propagate by budding out similar cells from their surface. The bud may become detached from the parent cell, or it may remain attached and itself produce another bud. In this way, a chain of cells may be produced. Under certain conditions, continued elongation of the parent cell before its buds results in a chain of elongated cells or pseudohypha. Many fungi, including some of medical importance, can exist in a mycelial or a yeast form depending on the environmental conditions.

1.2 Fungi as Human Pathogens

Among the 50,000 to 250,000 species of fungi that have been described, fewer than 200 have been associated with human disease. In general, these organisms are free living in nature and are in no way dependent on humans (or animals) for their survival. With few exceptions, fungal infections of humans originate from an exogenous source in the environment and

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are acquired through inhalation, ingestion, or traumatic implantation.

A handful of fungi are capable of causing significant disease in otherwise normal individuals. Many more are able to produce disease only under unusual circumstances, mostly involving host debilitation. However, as a result of the numerous developments in modem medicine, these hitherto inocuous organisms have gained increasing prominence as aetiological agents of disease. Any fungus capable of growing at the temperature of the host (37°C) and surviving in a lowered oxidation–reduction state (a situation found in damaged tissue) must now be regarded as a potential human pathogen.

Fungal infections can be classified into a number of broad groups according to the initial site of infection. Grouping the diseases in this manner brings out clearly the degree of parasitic adaptation of the different groups of fungi and the way by which the site affected is related to the route of the fungus entering the host.

1.3 Systemic Mycoses

These are infections that usually originate in the lungs, but may spread to many other organs. These infections are most commonly acquired as a result of inhaling spores of organisms that grow as saprotrophs in soil or decomposing organic matter, or as pathogens on plants.

The organisms that cause systemic fungal infection can be divided into two distinct groups: the true pathogens and the opportunists. The first of these groups consists of a handful of organisms, such as *Histoplasma capsulatum* and *Coccidioides immitis*, which are able to invade and develop in the tissues of a normal host with no recognizable predisposition. Often these organisms possess unique morphological features that appear to contribute to their survival within the host. The second group, the opportunists, consists of less virulent and less well adapted organisms, such as *Aspergillus fumigatus*, which are only able to invade the tissues of a debilitated or immunocompromised host.

In most cases, infections with true pathogenic fungi are asymptomatic or mild and of short duration. Many cases occur in regions endemic for the fungus and follow inhalation of spores that have been released into the environment. Individuals who recover from these infections enjoy marked and lasting resistance to reinfection, while the few patients with chronic or residual disease develop a granulomatous response.

In addition to their well-recognized manifestations in normal individuals, infections with true pathogenic fungi have emerged as important diseases in immunocompromised individuals. Histoplasmosis and coccidioidomycosis, for instance, have been recognized as AIDS-defining illnesses. Both diseases are now being seen in significant numbers of AIDS patients in parts of North and South America. In debilitated or immunocompromised patients, infections with true pathogenic fungi are often life-threatening and unresponsive to antifungal treatment, or relapse following treatment resulting in death.

Opportunistic fungal infections occur in individuals who are debilitated or immunosuppressed as a result of an underlying disease or their treatment. In most cases, infection results in significant disease. Resolution of the infection does not confer protection, and reinfection or reactivation may occur if host resistance is again lowered. Many of the organisms involved are ubiquitous saprotrophs, found in the soil, on decomposing organic matter, and in the air. Although new species of fungi are regularly being identified as causes of disease in immunocompromised patients, four diseases still account for most reported infections: aspergillosis, candidosis, cryptococcosis and mucormycosis (zygomycosis.) (Jantunen et al. 1997; Ribaud 1997; Toren et al. 1997; Tumbarello et al. 1997; Walsh et al. 1996). In neutropenic patients, these infections are a major cause of morbidity and mortality. Between 20 and 40% of mycoses in patients with hematological malignancies are disseminated, and more than 70% of these are fatal. Candidosis and aspergillosis are the most common fungal infections in patients receiving immunosuppressive therapy and organ transplantation (Richardson and Kokki 1998). Late-onset *Pneumocystis carinii* pneumonia is also seen (Lyytikainen et al. 1996).

Many of the systemic fungal infections of humans have a restricted geographical distribution, being limited to regions where the causal organisms are found in nature. For instance, *C. immitis* only occurs in the soil in certain semi-arid parts of southwestern North America, and similar regions in Central and South America, where there are hot summers and few cold periods in winter. Most cases of human coccidioidomycosis are acquired in these regions. On the other hand, *Cryptococcus neoformans* is found wherever there are bird droppings, and cases of cryptococcosis occur throughout the world.

In contrast to the restricted geographical distribution of most of the true pathogenic fungi, the spores of many opportunistic fungi are ubiquitous in the environment and often reach high concentrations in hospital air. Nosocomial (hospital-acquired) outbreaks of aspergillosis and other infections have become associated with hospital construction or renovation work in or near units where immunosuppressed patients are housed. These outbreaks have highlighted the need for efficient ventilation with filtered air in such units and for careful surveillance.

1.4 The Changing Pattern of Fungal Infection

Over the past few years, improvements in the management of debilitated medical and surgical patients have led to an unwelcome increase in the number of life-threatening infections due to true pathogenic and opportunistic fungi. These infections are being seen in ever-increasing numbers

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among cancer patients, transplant recipients, and patients receiving broad spectrum antibiotics or parenteral nutrition. Fungal infection is also becoming more common among other groups of debilitated or seriously ill patients, such as drug addicts and patients with AIDS. Estimates of the incidence of these infections are thought to be quite conservative in comparison with their true magnitude, because many fungal infections go undiagnosed.

In addition to the rise in opportunistic fungal infections due to such well-recognized organisms as *A. fumigatus* and *Candida albicans*, an ever-increasing number of fungi, hitherto regarded as harmless saprotrophs, are being reported as the cause of serious or lethal infection in immunocompromised individuals. For instance, *Trichosporon beigelii*, the aetiological agent of the mild dermatological condition white piedra, is now well documented as a cause of lethal disseminated infection in neutropenic cancer patients and bone marrow transplant (BMT) recipients. The emergence of this organism as a significant pathogen has important implications for diagnosis and management, because the clinical presentation can mimic candidosis but the organism is often resistant to the drug (amphotericin B) used to treat that infection. The saprotrophic soil mould *Scedosporium apiospermum* is another organism that can cause life-threatening infection that mimics a more common condition, aspergillosis. It, as well, is often resistant to amphotericin B, the drug of choice for aspergillus infection.

In certain respects the changing pattern of fungal infection in the developed world is quite different from that seen in the developing world. Throughout the developed world, acquisition of resistance to azole antifungal has become a major issue in AIDS patients given long-term treatment to suppress persistent oral infection with *C. albicans*. Another significant development has been an apparent rise in the prevalence of species other than *C. albicans* as agents of serious deep-seated Candida infection. The precise reasons for this increase are difficult to pinpoint, but changes in medical practice, such as the widespread use of triazole antifungal compounds as prophylactic and therapeutic agents in neutropenic cancer patients and transplant recipients, seem to be important factors in the selection of unusual drug-resistant organisms such as *Candida glabrata*.

In the developing world, the AIDS epidemic has led to a dramatic increase in the number of deaths from systemic fungal infection, and from histoplasmosis and cryptococcosis in particular. In parts of Africa, the incidence of cryptococcosis has risen to more than 30% in patients with AIDS and the number of cases is increasing in Asia and South America. In parts of South East Asia the dimorphic mould *Penicillium marneffei* has emerged as the third most common opportunistic infection, after tuberculosis and cryptococcosis. There has also been an unprecedented rise in the number of cases of *P. marneffei* infection diagnosed in European, North American and Australian AIDS patients, infected during visits to the endemic region.

1.5 Implications for Diagnosis and Management

The dramatic increase in the number and range of different fungal infections now being reported has been due to a combination of improved recognition and an increasing population of susceptible patients. This rise in prevalence has resulted in an increased awareness of the need for improved methods of diagnosis and for new methods of management. As with other microbial infections, the diagnosis of fungal disease is based on a combination of clinical observation and laboratory investigation.

Laboratory methods for the diagnosis of fungal infection continue to be updated, but for the most part depend on isolation of the fungus in culture, on its detection in clinical material by direct microscopic examination, and on the detection of an immunological response to the pathogen or some other marker of its presence, such as a metabolic product. Now that an increasing number of common culture contaminants (and other less usual environmental moulds) are occurring as occasional opportunistic pathogens, it is more important than ever that medical microbiologists should be able to recognize these organisms, at least to genus level. The application of molecular biological techniques to the identification of fungi is attracting attention, and while practical procedures have been developed for distinguishing strains of particular organisms, it is doubtful whether these methods can supplant the traditional morphological approach to mould identification.

Molecular biological techniques have also been developed for the detection of fungal pathogens in clinical specimens [see a recent review by Chen et al. (2002)]. In most cases, fungal DNA has been detected following amplification by the polymerase chain reaction (PCR). Species-specific primers have been designed for a number of organisms, including *A. fumigatus*, *C. albicans* and *C. neoformans*, and several have been used in attempts to detect fungal DNA in specimens, such as blood, urine, and cerebrospinal fluid.

Monoclonal antibodies to structural components of the major fungal pathogens of humans are now being produced. These reagents have the potential to form the basis for new tests for identification of organisms. Their introduction has stimulated significant developments in the diagnosis of fungal infection, by enabling improved tests, for the detection of circulating fungal antigens in immunocompromised patients, to be devised. Monoclonal-based serological tests are now being marketed for the diagnosis of deep candidosis and aspergillosis, as well as for cryptococcosis.

The increased prevalence of life-threatening fungal infection has stimulated interest in the development of new antifungal drugs. New agents, such as the triazoles and the allylamines, have been introduced and new formulations of older compounds, such as lipid-based forms of amphotericin B, have appeared. These developments have improved the treatment of many forms of fungal infection, but problems remain. There are still important infections, such as mucormycosis, for which no reliable treatment has been

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developed. Then again, many strains of the unusual organisms, such as *Candida krusei* and *T. beigelii*, which are now being isolated from debilitated patients are insensitive to current antifungal compounds.

Unfortunately, a major obstacle to the successful treatment of invasive fungal infections is the lack of sensitive and specific methods for the early diagnosis of invasive fungal infections. Standard approaches to the laboratory diagnosis of invasive fungal infections include (a) direct microscopic visualization for the presence of organisms in freshly obtained body fluids, (b) histopathologic demonstration of fungi within tissue sections, and (c) cultivation of the causative fungus and its subsequent identification. However, these approaches often are not sufficiently sensitive and/or specific to diagnose invasive fungal infections, and they sometimes require invasive procedures to obtain the necessary specimens.

This article reviews recent advances of nonculture methods for the diagnosis primarily of invasive aspergillosis (IA) and systemic candidosis. Among the nonculture methods reviewed, detection of a specific host antibody response is attractive because such tests can be performed rapidly and do not require invasive sampling procedures. However, the presence of host antibodies does not always correlate with presence of invasive disease, especially in patients whose abilities to produce specific immunoglobulin responses may be impeded by immunosuppressive drugs and/or serious underlying diseases. Detection of macromolecular microbial antigens generally requires a relatively large microbial burden that may limit assay sensitivity. Nonetheless, several examples of successful antigen detection systems have been developed, and some of these are widely used. Other alternatives to standard culture and serologic diagnostic methods include amplification and detection of specific fungal DNA sequences and the detection and quantitation of specific fungal metabolic products.

2 DIAGNOSIS OF SUSPECTED FUNGAL INFECTION

The diagnosis of fungal infection in immunocompromised patients remains a major difficulty for the clinician. Recognizing this problem, a series of recommendations have been drawn up to provide guidance to clinicians, microbiologists, and infectious disease physicians, which incorporate currently available diagnostic techniques (Denning et al. 1997; Richardson and Warnock 2003). The present review highlights the key ongoing developments in the design of immunoassays and molecular methods. The reader is also recommended to consult recent exhaustive reviews that expand many of the concepts and achievements given here (Buchheidt et al. 2000; Walsh and Chanock 1997; Yeo and Wong 2002).

The laboratory diagnosis of fungal infection in the immunocompromised patient involves one or more of four approaches: (a) a thorough examination of respiratory secretions by direct microscopy, (b) isolation of the organism, (c) detection of antibody or antigen, and (d) histopathological evidence of invasion (LaRocco and Burgert 1997; Yuen and Woo 1997). The isolation of fungi from otherwise sterile sites provides vital information to the clinician. Sputum cultures that yield *Aspergillus* species should be considered significant unless proved otherwise; however, expectorated sputum is generally of little help in the diagnosis of fungal infections. Where there is diffuse disease, bronchoalveolar lavage (BAL) or bronchial biopsy should provide a more reliable and accurate means of diagnosing pulmonary infection and may be positive in up to 60% of cases. Culture is invariably negative where there are focal lung lesions. Here, radiographically guided fine-needle biopsy may be helpful. However, if a BAL fluid is the only specimen available it should be screened very extensively for fungal elements using direct microscopy and Calcofluor white staining. Until recently, serological tests have not been particularly successful in the early diagnosis of invasive disease in transplant recipients. Tests designed to detect circulating protein and carbohydrate antigens of *Candida* have been extensively evaluated but their value remains controversial. Although the detection of circulating antigen may correlate with IA, the routine use of antigen detection tests is still to be accepted.

The problem of designing new immunoassays for the detection of fungal components in clinical specimens has been approached from many directions: detection of metabolites, e.g., β-1,3-D-glucan; detection of enzymes, e.g., enolase; detection of cell wall components, e.g., galactomannan, mannoprotein; detection of DNA; serology to detect antibody responses to whole-cell lysates or specific antigens.

3 INVASIVE ASPERGILLOSIS AND ITS DIAGNOSIS

Invasive aspergillosis is one of the most common fungal infections in hematological neoplastic disease (14–20% of cases) (Latge 1999; Leung et al. 1999; Patterson et al. 1997; Salonen and Nikoskelainen 1993; Wald et al. 1997; Williamson et al. 1999). The infection primarily affects the lungs. Among the patient population, at greatest risk for infection are those with inadequate numbers of circulating neutrophils and those with defective neutrophil function. The crude mortality for patients with IA is up to 95%, which is partly due to the difficulty of diagnosing the infection at an early stage of disease. More effective preventative and prophylactic measures would reduce the incidence of this disease.

Predisposing factors must be taken into account when assessing the risk of acquiring IA. Because of the difficulty of diagnosing IA and because of its rapid progression (1–2 weeks from onset to death) and severity, clinicians often treat the patient empirically rather than waiting for the diagnosis to

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be established. Moreover, waiting until the diagnosis is confirmed subjects the patient to a greater risk of untreatable IA, since the fungal burden might reach a level too high for antifungal therapy.

3.1 Clinical Signs and Symptoms

The presentation of acute pulmonary aspergillosis often mimics an acute bacterial pneumonia. Cough, usually unproductive, and fever are the most frequent presenting symptoms. Pleuritic chest pain is common and a pleural friction rub is not unusual. Pleuritic pain in a leukemic patient with persistent cough and fever suggests IA even in the absence of a chest x-ray abnormality.

Cavitation is strongly suggestive of invasive pulmonary aspergillosis in neutropenic patients. Computed tomography (CT) scanning of the chest has made a major impact on the management of this patient group (Caillot et al. 1997; Graham et al. 1991). It is more sensitive than chest radiography and is particularly valuable when the chest x-ray is negative or shows only subtle changes. In one study, intrathoracic complications of BMT were found using CT scanning in 57% of patients in whom the chest x-ray was negative (Graham et al. 1991). It can often differentiate between invasive pulmonary aspergillosis and bacterial and viral infections. Typical features of IA include wedge-shaped peripheral areas of consolidation, usually extending to the pleural surface, with or without cavitation, or nodular areas of consolidation, often related to blood vessels. Computed tomography scanning is also helpful in guiding further invasive diagnostic procedures, such as the best location for needle biopsy or open lung biopsy, or in defining whether bronchoscopy is the best modality for confirming diagnosis.

3.2 Antibody and Antigen Detection

Confirmation of the diagnosis of *Aspergillus* infection ideally requires culture of tissue from deep sites; invasive procedures to obtain this type of clinical specimen are difficult to perform in neutropenic and thrombocytopenic patients. Serological methods, including the detection of antibodies to *A. fumigatus* may be a useful aid in the diagnosis of some clinical forms of aspergillosis. The usefulness of antibody detection in IA may become clearer now that new commercial kits are available for individual immunoglobulin classes. These kits are currently being evaluated in bone marrow recipients. However, in presumptive cases of IA, current practice tends towards the detection of *Aspergillus* antigens, since antibody tests may be negative because of the poor immune status of patients. In some patients, *Aspergillus* antigen tests (latex agglutination and ELISA (enzyme linked immunosorbent assay)) have proved to be useful for the detection of antigen in a range of body fluids (Verweij et al. 1996). Some are commercially available. The *Aspergillus* antigen galactomannan can be detected in plasma or serum samples from patients with IA by the commercially available sandwich ELISA (Platelia *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). This assay has a reported sensitivity of 67–100% and a specificity of 81–98% when performed with sera from patients receiving treatment for hematological malignancies (Verweij et al. 1996).

The sandwich ELISA for the detection of galactomannan is currently the most sensitive method developed. Moreover, the analysis takes only 2 h to perform. Several studies performed in Europe have shown that the sandwich ELISA contributes to the early diagnosis of IA, and the inter- and intralaboratory reproducibility of the method is reasonably good. Galactomannan appears to be detected in all specimens following the first positive specimen during the course of disease in a given patient. Although it is known that the highest concentration of galactomannan is always released in the terminal phases of the disease, the pharmacokinetics of the antigen in infected animals or humans has been insufficiently studied. Depending upon the patient, positive antigenaemia can last from 1 week to 2 months. Galactomannan is detected at a lower concentration in urine (0.5 ng/ml) than in serum (1.0 ng/ml). However, the presence of antigen in urine has been shown to be inconsistent and, when present, it does not occur before antigen could be detected in serum. Therefore, serum appears to be the most appropriate specimen for the detection of galactomannan in IA. Interestingly, galactomannan can also be detected earlier in BAL samples than in serum, but this sampling method is not always possible in IA patients. Furthermore, galactomannan can be detected in the cerebrospinal fluid of patients with cerebral aspergillosis (Viscoli et al. 2002). The ELISA for detection of galactomannan becomes positive at an early stage of infection. Early detection is probably the most important feature of this assay, because the detection of antigenaemia dictates the initiation of therapy. In some patients, galactomannan is detected in serum before signs and symptoms consistent with IA become apparent. Recent studies have shown that IA may be treatable with amphotericin B if diagnosed at this stage (Bretagne et al. 1997; Rohrlich et al. 1996). Another advantage of the ELISA is the possibility that antigen titres in serum can be monitored during treatment. A decrease in the concentrations of galactomannan in serum is indicative of a response to treatment (Patterson et al. 1988; Rohrlich et al. 1996; Tabone et al. 1997; Verweij et al. 1997).

Both antigenaemia and antigenuria are transient in nature. However, the highly sensitive methods now available allow detection of very low level of antibodies in serum samples. For example, using analytical isoelectrofocusing in conjunction with immunoblotting, 11 of 13 patients with proven or highly probable cases of IA had anti-*Aspergillus* IgG to multiple antigenic preparations of *A. fumigatus* (Hearn et al. 1995). This study shows that this type of technique is highly sensitive and specific for IA. However, it also indicates that the use of a spectrum of antigenic fractions is advisable, given the variability observed in the immune response of individual patients.

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3.3 Polymerase Chain Reaction

PCR methods for the detection of *Aspergillus* DNA fragments in body fluids are currently being developed and evaluated [reviewed recently in Chen et al. (2002)]. Selected illustrative studies are presented here. Positive results have been reported in urine and BAL fluid samples from patients suspected of having IA (Verweij et al. 1996). However, positive amplification reactions have also been observed in 6–23% of BAL fluid samples from patients without invasive infection, which may limit the diagnostic value of this test (Yamakami et al. 1996). Recently, very promising PCR results were obtained with serum or plasma (Bretagne et al. 1998; Einsele et al. 1997; Van Burik et al. 1998).

Another approach for the PCR diagnosis of IA involves the use of universal primers that target the conserved sequences of ribosomal RNA (rRNA) genes common to all fungi (Hopfer et al. 1993; Sandhu et al. 1995). The 18S and 28S rRNA subunits contain intervening variable domains, and the rRNA subunits are separated by highly variable internal transcribed spacer regions (ITS1, ITS2). These variable sequences can be exploited for family or species identification by nested amplifications, restriction fragment length polymorphism analysis, hybridization with specific probes, single-strand conformation polymorphism analysis, or the sequences may be differentiated by length. A further development is where two characteristics of the variable sequences have been combined in the detection of pathogenic fungi, including *Aspergillus* (Hendolin et al. 2000). First, the ITS regions including the 5.8S rRNA gene are amplified with universal primers, and the amplification products are hybridized with species-specific probes. Then, the hybridized products are electrophoretically separated for identification by length. The use of product length for discriminatory criterion allows combining of probes, which reduces the number of simultaneous or sequential hybridizations for different species. Products that do not hybridize with the selected probes can be identified by sequencing. The method is able to detect less than 1 pg (10–12 g) of fungal DNA (50 organisms) and it can be completed in two working days. Sequencing of the products prolongs the detection time by an additional day. Currently, the method is being tested with clinical specimens.

The choice of clinical specimen is currently being determined (Loeffler et al. 2002). For example, the usefulness of a nested PCR assay for detection of *Aspergillus* sp. DNA was evaluated in 177 BAL fluid specimens. This test was accurate both to diagnose culture-negative BAL fluid specimens from patients with invasive pulmonary aspergillosis and to confirm culture-positive samples. However, it did not differentiate between infection and colonization (Hayette et al. 2001).

The use of PCR technology with serum or whole blood should be pursued, since it has several advantages over the use of BAL samples (Kami et al. 2001). First, assuming appropriate handling of the specimen, false positive results do not occur from environmental contamination. Second, obtaining blood is considerably easier than obtaining BAL fluid. Third, sampling can be repeated, so that PCR quantification can be done along with ELISAs. Compared to ELISA, however, PCR positivity seems to occur later than galactomannan detection (Bretagne et al. 1998). However, the combined use of PCR and ELISA should result in a definitive diagnosis of IA, even in the absence of obvious clinical signs (Costa et al. 2002). Finally, PCR data raise an interesting question as to the origin of the *A. fumigatus* DNA, since the organism is not usually cultured from blood, even in the late stages of disease.

A variety of quantitative protocols have been developed. For example, oigonucleotide primers and a fluorescently labeled probe have been designed for use with quantitative polymerase chain reaction (QPCR) (Cruz-Perez et al. 2001. Primers and probe were tested for selectivity, specificity, and sensitivity of detection of the target organism using a fluorogenic nuclease assay and a sequence detector. The DNA extraction protocol consisted of enzymatic treatment and boiling of fungal spore suspensions followed by DNA concentration and purification. The primer set developed was specific for *A. fumigatus* and had a sensitivity of <20 template copies. These primers amplified all *A. fumigatus* isolates tested and did not amplify DNA extracted from other *Aspergillus* spp. or 15 other fungal genera. However, one *A. fumigatus* sample was initially negative after PCR amplification. Incorporation of an internal positive control in the PCR reaction demonstrated the presence of inhibitors in this and other samples. The PCR inhibitors were removed by dilution or further purification of the DNA samples. This research resulted in a QPCR method for detection and quantitation of *A. fumigatus* and demonstrated the presence of PCR inhibitors in several *A. fumigatus* isolates. Molecular techniques are ideally suited to prospective screening of high-risk patients for either aspergillosis or candidosis. To determine whether a PCR-based assay enabled the identification of patients at risk for invasive fungal infections, a prospective monitoring once per week was performed during 92 neutropenic episodes in patients receiving chemotherapy for acute leukemia or high-dose therapy followed by allogeneic or autologous stem cell transplantation, with the investigators blinded to clinical and microbiological data (Hebart et al. 2000a). The PCR positivity was documented in 34 out of 92 risk episodes. All patients developing proven invasive fungal infection were found PCR positive, and PCR was found to be the earliest indicator of invasive fungal infection preceding clinical evidence by a mean of 5.75 days (range 0–14 days). In febrile neutropenic patients without a prior history of invasive fungal infection, a sensitivity of 100% and a specificity of 73% of the PCR assay for the development of proven or probable invasive fungal infection was documented. In conclusion, panfungal PCR performed prospectively once a week enabled the identification of patients at high risk for invasive fungal infections.

To assess specifically whether prospective PCR screening could facilitate the early diagnosis of IA, 84 recipients of an allogeneic stem cell transplant were analyzed with

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the investigators blinded to clinical and microbiologic data (Hebart et al. 2000b). Of 1193 blood samples analyzed, 169 (14.2%) were positive by PCR. In patients with newly diagnosed IA (n = 7), PCR positivity preceded the first clinical signs by a median of 2 days (range, 1–23 days) and preceded clinical diagnosis of IA by a median of 9 days (range, 2–34 days). Pretransplantation IA [relative risk (RR), 2.37], acute graft-vs.-host disease (RR, 2.75), and corticosteroid treatment (RR, 6.5) were associated with PCR positivity. The PCR assay revealed a sensitivity of 100% [95% confidence interval (CI), 48–100%] and a specificity of 65% (95% CI, 53–75%). None of the PCR-negative patients developed IA during the study period. Thus, prospective PCR screening appeared to identify patients at high risk for subsequent onset of IA.

In a 2-year study, 121 patients admitted to the University Hospital of Innsbruck for cancer chemotherapy without clinical signs of fungal infection were prospectively screened for *Aspergillus* spp. (Lass-Florl et al. 2001. In 28 out of 121 (23%) patients, *Aspergillus* DNAaemia was detected. Of these patients, 16 (57%) were positive only once for *Aspergillus* DNA, but positivity was never associated with IA. The PCR positive episodes were short and resolved without antifungal treatment. Five patients (18%) had intermittent PCR positive results. Seven (25%) patients presented at least two consecutive positive PCR results; one of these patients developed IA and another two were strongly suspected as having aspergillosis. Based on the criteria of the European Organization for Research and Treatment of Cancer case definitions, sensitivity, and specificity of serial PCR monitoring were 75 and 96%. Positive PCR results became negative shortly after commencement of antifungal treatment, but the changes did not correlate with clinical responsiveness to treatment in three patients. The results indicate the potential usefulness of PCR for screening for *Aspergillus* spp. in patients at risk, but without antifungal treatment (Lass-Florl et al. 2001).

Nucleic acid sequence-based amplification (NASBA), an isothermal amplification technique, was established and evaluated for the detection of *Aspergillus* RNA and compared with a previously published, well-defined real-time PCR assay amplifying a region of the *Aspergillus* 18S rRNA gene. The NASBA showed a lower detection limit of 1 CFU and detected RNA from five different clinically relevant *Aspergillus* spp., including *A. fumigatus*. All 77 blood samples tested by PCR and NASBA showed identical results in both assays. Results with the NASBA technique were obtained within 6 h. Thus, the NASBA technique provided a valuable tool for sensitive, specific, fast, and reliable detection of *Aspergillus* RNA with potential for routine diagnosis, including the possibility to test the viability of cells (Loeffler et al. 2001).

3.4 The G-Test (Glucatell)

The cell wall of *Aspergillus* hyphae, and the cell walls of other pathogenic fungi, consist of mannans and glucans. The detection of circulating β-1,3-D-glucan is another investigative strategy for diagnosis of IA (Obayashi et al. 1995). The plasma concentration of β-1,3-D-glucan has been measured at the time of routine cultures in febrile episodes (Obayashi et al. 1995). With a plasma cut-off value of 20 pg/ml, 37 of 41 episodes of proven fungal infections (confirmed at postmortem or by microbiological methods), including IA, were detected. All of 59 episodes of non-fungal infections, tumor fever, or collagen-vascular diseases had concentrations below the cut-off value (specificity 100%). Of 102 episodes of fever of unknown origin, 26 had plasma glucan concentrations of more than 20 pg/ml. If these 102 cases are taken as nonfungal infections, the positive predictive value of the test was estimated as 59% (37 of 63), the negative predictive value as 97% (135 of 139), and the efficacy as 85% (172 of 202). Although a positive result does not indicate the specific cause of the detected invasive fungal infection, this approach is very encouraging and warrants more extensive investigation in selected patient populations. The test is available commercially in an ELISA format (Glucatell, Associates of Cape Cod Inc).

3.5 Clinical and Laboratory Diagnosis of IA in Practice

The proponents of *Aspergillus* antigen detection in the diagnosis of IA have formulated an approach to early detection and management of disease by including antigen testing by ELISA for galactomannan with the conventional diagnostic tools of thoracic CT and radionuclide imaging (Severens et al. 1997). This approach was compared with

Table 1 Molecules detected in biological fluids of patients with IA due to *A. fumigatus*

Antigen	Biological fluid	Detection limit
Galactofuran-containing antigens[a]	Serum, urine, BAL fluid	0.5–1.0 ng/mL
29, 18, 11 kDa[b]	Urine	?[c]
β-1,3-D-glucan	Serum	10–2

[a] Glycoprotein and polysaccharide.
[b] Plus other minor antigens.
[c] Unknown; detection by immunoblotting.

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a combination of clinical symptoms, persistent fever, and chest radiographic findings. Use of the strategy incorporating the antigen assay would appear to reduce the number of patients who would receive antifungal treatment empirically, especially if liposomal amphotericin B (AmBisome™) was going to be used.

In summary, the detection of various infection-specific markers of *A. fumigatus* infection is currently an area of great interest. The molecules detected in biological fluids, and the lower limit of detectability, are summarized in Table 1. However, it is virtually impossible to compare and contrast the different approaches because of different patient populations. Only where commercial kits are used can meaningful comparisons be made. The development of tests is encouraging but further commercial development and evaluations are needed. The current status of the availability of these new approaches is summarized in Table 2.

4 SYSTEMIC CANDIDOSIS AND ITS DIAGNOSIS

Candidosis is a spectrum of infections that may be classified as cutaneous, mucosal, and deep invasive. Deep invasive infection may be further classified as fungaemia, tissue-proven disseminated candidosis, and single-organ candidosis. Disseminated candidosis may be distinguished as acute and chronic disseminated candidosis, which constitute two ends of a clinical and pathological continuum. The types of mucosal candidosis that are commonly encountered in neutropenic patients are oropharyngeal and oesophageal disease.

The incidence of candidaemia in Europe and the United States has increased substantially in the past few years. Numerous risk factors for candidaemia have been identified. They vary among institutions but usually include use of antibiotics, indwelling catheters, hyperalimentation, cancer therapy, and immunosuppressive therapy after organ transplantation. Equally, the incidence of serious *Candida* infections is rising rapidly (Martino et al. 1994). For example, hepatosplenic candidosis was recognized as a distinct form of systemic candidosis in patients with acute leukemia in the early 1980s. The incidence of this form of disease seems to have increased. Although hepatosplenic candidosis is recognized as a major problem in the treatment of acute leukemia, there are little data on its incidence among patients with leukemia. In one tertiary care hospital where the incidence among 562 adult patients with acute leukemia during the period 1980 to 1993 was studied, 38 (6.8%) had hepatosplenic candidosis (Anttila et al. 1977a). The incidence of infection increased five-fold during the study period. Hepatic candidosis is associated with high mortality because clinical tests and imaging radiographs are relatively insensitive in identifying intra-abdominal fungal infection.

The difficulties for clinical diagnosis of systemic candidosis lie in the absence of specific clinical signs. Difficulties for mycological diagnosis lie in the opportunistic character of yeasts. Their presence in normally colonized body sites of immunocompromised patients does not prove infection, and they are rarely isolated from infected deep organs or tissues, including blood. If a confirmed diagnosis of candidosis is obtained in the early stages of infection and antifungal therapy is administered promptly, the prognosis can be improved dramatically. Unfortunately, the sensitivity of currently used blood culture methods and histological analysis of biopsy material in detecting *Candida* spp. is relatively low, especially early in infection. This is due to the slow growth of many fungal species, with the result that it can take from 2 to 2 days (and often longer) for the species of the infecting organism to be cultured and identified correctly. In addition, in many cases the infecting organisms may be nonculturable or present in low numbers. Consequently, many cases of systemic candidosis are often diagnosed too late to save the patient and in some cases are only diagnosed postmortem. Furthermore, in cases of a rapidly progressing disease, antifungal therapy is often given empically before a definitive diagnosis has been made. This approach is expensive.

To decrease the time required to diagnose fungal infections and therefore reduce mortality associated with systemic candidosis, a number of different strategies based on molecular tools have been adopted, each with its own specific advantages and disadvantages. Among the most promising of the techniques that have been adapted and applied to the rapid identification of *Candida* spp. in clinical samples are molecular biological techniques that allow the detection of Microbiol nucleic acid sequences directly in blood or biopsy material.

Establishing the diagnosis of deep-seated candidosis is difficult because the clinical presentation is nonspecific and the results of microbiological and serological tests are difficult to interpret (Walsh and Chanock 1997). In cases of suspected deep-seated candidosis, cultures should be made from as many sources as possible and efforts should be made to obtain material for histopathological examination. An illustrative point is made in the case of hepatosplenic candidosis. Culture of blood and tissue is frequently negative. Although imaging methods reveal typical foci in the liver and spleen, in many cases both culture and microscopical examination of biopsy specimens remain negative for fungi

Table 2 Immunoassays for the diagnosis of IA

Marker	Method	Availability
IgA, IgG, IgM	ELISA	Commercial kits
Galactomannan	ELISA	Commercial kits
Glucan	Calorimetric	Commercial kits
Glucan	ELISA	Investigational
DNA	PCR	Investigational

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(Anttila et al. 1977a). However, laparoscopy-guided liver biopsy appears to enhance the diagnosis of suspected hepatosplenic candidosis in leukemic patients (Anttila et al. 1997b). *Candida* was detected more often if the laparoscopy was performed during the 3-week period after recovery from neutropenia than when performed later.

Despite advances in blood-culture technology, the extent to which detection of candidaemia establishes the presence of deep invasive candidosis is limited. Little is known about the sensitivity of current blood-culture methods in detecting systemic disease. A number of approaches have been investigated.

4.1 The G-Test (Glucatell)

As with cases of IA high levels of glucan have been found in patients with candidaemia and other forms of systemic candidosis (Obayashi et al. 1995).

4.2 Arabinitol Detection

Many publications have indicated the clinical usefulness of arabinitol determination by gas liquid chromatography in the diagnosis of systemic candidosis (reviewed in Christensson et al. 1999; Walsh and Chanock 1998). Recent refinements include the distinction between arabinitol of fungal and nonfungal origin in urine (Lehtonen et al. 1996; Salonen et al. 2001). Using this approach the diagnosis of disseminated infection in 17 patients with acute leukemia was confirmed on average 21.7 days after the first elevation of the D-arabinitol (fungal origin) and L-arabinitol (part of normal human metabolism) ratio. A more recent report underscores the usefulness of determining arabinitol in urine (Christensson et al. 1997. Positive D-arabinitol/L-arabinitol ratios were found for all of 10 children with confirmed invasive candidosis. D-arabinitol/L-arabinitol ratios were positive 3–31 days (median 12 days) before the first positive blood culture or empirical therapy was initiated. The conclusion from this work was that the regular monitoring of D/L-arabinitol ratios in urine held great promise for diagnosing systemic candidosis in immunocompromised children.

4.3 Antigen Detection

Immunoassays for the detection of *C. albicans* protein antigens of 47 and 48 kDa have represented promising advances [reviewed in Walsh and Chanock (1998) and Yeo and Wong (2002)]. Unfortunately, the use of some of the commercially available kits has been restricted by their prohibitive cost. The Directigen 1-2-3 kit for the 48-kDa *Candida* enolase marker appeared to be very promising (Walsh et al. 1991) but it is no longer available. In contrast, the Cand Tec latex agglutination antigen detection test has been widely used as the first commercially available antigen detection test but the still unknown nature and function of the target antigen has impeded its acceptability or further development (Herent et al. 1992; Örmälä et al. 1995).

4.4 Detection of Anti-*Candida* Antibodies

As diagnostic markers of systemic candidosis, anti-*Candida* antibodies have been studied extensively since the 1960s. These assays were developed as precipitin tests or passive hemagglutination techniques using crude cell wall or cytoplasmic antigens. Due to problems of specificity and sensitivity these assays have failed to contribute to the diagnosis of systemic candidosis. However, more recently there has been a resurgence of interest in antibody detection, including tests designed to monitor antibodies to germ tubes of *C. albicans* (Garcia-Ruiz et al. 1997) and enolase (discussed earlier) (Deventer van et al. 1994; Mitsutake et al. 1994). Currently, a number of commercial ELISA kits have been developed to detect a range of immunoglobulin responses to *Candida*. These are undergoing evaluation in various populations of immunocompromised patients (Sendid et al. 1999; Sendid et al. 2002).

4.5 Combinations of Tests

It is hoped that using a combination of these tests will provide an early, specific diagnosis of systemic candidosis. An interesting feature of the serological detection of *C. albicans*-derived antigens in patient sera, in contrast to the detection of nonimmunogenic molecules, is that the detected molecules may elicit an antibody response in infected patients. Depending on the nature of the antigen and its relevance to infection, joint consideration of antigenaemia and antibody response may provide an insight into the progression and nature of infection. For example, patients with systemic candidosis have been shown to be positive for enolase and antienolase (unpublished data). Although not a new idea, the concept of a combination of tests is the basis of the latest commercial ELISA kits for the detection of *Candida* mannoprotein and anti-*Candida* immunoglobulin (Sanofi Diagnostics Pasteur, licensed to Bio-Rad). The antigen test (Platelia *Candida* Ag) detects mannan with a sensitivity of 0.1 ng/ml. The anti-*Candida* test (Platelia *Candida* Ab) detects antimannan antibodies. These tests have been evaluated with 162 serum samples retrospectively selected from 43 patients with mycologically and clinically proven candidosis (mainly candidaemia) (Sendid et al. 1999). Forty-three samples were positive for mannan and 63 had significant antibody levels. Interestingly, only five serum samples were simultaneously positive for both tests. When the results for each patient were analyzed, 36 (84%) had at least one serum sample positive by one test. For 30 of the patients, positivity by one test was always associated with negative results by the other test for any of the tested sera. The sensitivities

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and specificities were 40 and 98% and 53 and 94% for mannanaemia or antibody detection, respectively. These values reached 80 and 93%, respectively, when the results of both tests were combined. These observations, which clearly demonstrate a disparity between mannan circulation and detectability and an antimannan antibody response, suggest that use of both ELISAs may be useful for the routine diagnosis of systemic candidosis. Furthermore, these tests take only 2 h to perform. In a small retrospective study, sera from four patients with hepatosplenic candidosis were mannan negative but strongly positive for antimamran (unpublished observations). Other commercial tests are available for the detection of individual immunoglobulins to *Candida*. All of these new tests await further evaluation.

A further evaluation of the Platelia Candida ELISA kits has been carried out using sera selected retrospectively from intensive care and hematology patients with clinically suspected systemic candidosis, and from whom *Candida* spp. had been isolated from normally sterile sites (Sendid et al. 2002). Of the 21 patients infected with C. albicans, 13 had positive antigenaemia and 14 had a positive antibody response, including eight patients who were antigenaemia negative. The sensitivity of the combined tests was 100%. In patients infected with *C. glabrata* (n = 12) or *C. tropicalis* (n = 10), the sensitivity was 83 and 80%, respectively. For the remaining patients, infected with *C. parapsilosis* (n = 10), *C. krusei* (n = 8) or *C. kefyr* (n = 2), the sensitivity of the combined tests was 40, 50, and 50%, respectively. At least one of the serological tests was positive before yeast growth occurred in 60% of patients for whom a serum sample was available before blood culture sampling. An increase in serological test positivity to $>80\%$ was observed for sera obtained around the date of positive culture, irrespective of the *Candida* spp. isolated. These results suggest that regular serological monitoring for both mannanaemia and antimannan antibodies in at-risk patients may contribute to the early diagnosis of candidosis.

4.6 Molecular Diagnosis of Candidosis

The molecular diagnosis of candidosis has developed in a number of directions. Three broad approaches are currently being explored: (a) early and rapid detection of *Candida* in clinical specimens, (b) rapid genus or species identification either directly or from a positive culture, and (c) monitoring of drug susceptibility and response to therapy. Readers are encouraged to consult excellent recent reviews (Chen et al. 2002; Sullivan and Coleman 2002). The published methods encompass many different genomic amplification protocols, various DNA extraction methods, the advantages of either narrow- or broad-target primers, uni- or multicopy primers, and amplicon detection formats. Although the sensitivity is described as one CFU or 10–20 fg of yeast DNA, there is very little work on clinical specimens. One of the many developments in the molecular diagnosis of systemic *Candida* infections is the development of a PCR method to amplify a 350-bp segment of the P-450 lanosterol 14 α-demethylase gene (Morace et al. 1997. By restriction enzyme analysis (REA) of the resultant amplicons, this approach was used to identify *Candida* spp. most commonly involved in human infection. In preliminary studies, PCR–REA was capable of detecting as little as 200 fg of *Candida* DNA. The method is reported to detect as few as five CFU of *Candida* spp. per ml of blood. In a clinical evaluation, this method has been applied to patients who had hematological malignancies, neutropenia, and fever (Morace et al. 1999). Daily blood samples from 72 patients were analyzed by conventional blood culture and PCR–REA. Thirty-one patients were PCR–REA positive and four of the patients were also culture positive, The ultimate diagnosis of 13 of these patients and one patient who was PCR–REA negative was disseminated candidosis. The PCR method appears to be more sensitive than conventional blood cultures and has a high negative predictive value (97.5%) for the development of disseminated candidosis in neutropenic patients. Another issue concerning the development of PCR methods for the diagnosis of systemic candidosis is the choice of biological sample. Using a nested PCR assay to amplify specific targets on the rRNA genes of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. glabrata*, it was shown in a rabbit model of candidaemia that serum was the sample of choice (Bougnoux et al. 1999).

4.7 Comparison of PCR and Culture-Based Methods

Theoretically, PCR offers great potential for providing the basis of a rapid and sensitive diagnostic tests for systemic candidosis. However, has it lived up to its potential? Since the late 1980s there have been many studies investigating the efficacy of this technology in the diagnosis of *Candida* infections. In conventional PCR tests in which human and rabbit blood samples have been spiked with defined numbers of *Candida* cells, the sensitivity observed in most studies was in the region of 100 cfu/ml. In similar experiments, using enhanced PCR product detection methods (e.g., Nested PCR, hybridization, and EIA) the sensitivity can be increased by at least a factor of 10. In some studies as few as one or two cells or femtogram amounts of DNA/ml were detected (reviewed in Sullivan and Coleman 2002). In studies using animal infection models or clinical samples, PCR has been shown to be at least as efficient as culture at detecting circulating *Candida* cells (reviewed in Sullivan and Coleman 2002). In another study in which patients deemed to be at risk of candidaemia were assessed, four out of nine patients who were culture-negative yielded positive PCR results. One of these PCR-positive patients subsequently became culture-positive 7 days later, thus indicating the potential of PCR to allow early diagnosis in individuals at risk. One complication, however, is that PCR also has the ability to detect *Candida*

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DNA or nonviable cells in blood, this leading to positive test results that would prove negative on culture.

4.8 Clinical and Laboratory Diagnosis of Candidosis in Practice

Patient evaluation, surveillance cultures, blood cultures, diagnostic imaging, and biopsies (as a source for histopathology and culture of tissue) are the standard clinical and laboratory approaches in diagnosis and therapeutic monitoring of systemic candidosis. Unfortunately, in many cases these methods lack sensitivity in the early recognition of this infection and are imprecise as markers of complete eradication of infection.

The practical diagnosis of candidosis still very much relies on clinical data, multiple cultures, histology of biopsy or postmortem specimens, and/or ultrasonographic evidence of hepatosplenic candidosis. The introduction of new sensitive antigen/antibody detection systems will most probably improve the diagnostic dilemmas. Early detection by these methods or by, in the longer term, PCR assays should improve the survival of patients with hematological malignancies by guiding the intervention with antifungal treatment while the fungal biomass is still low. Any new immunoassay or DNA detection method for systemic candidosis must distinguish between cutaneous/mucocutaneous and systemic disease. Furthermore, there has to be a greater understanding of the expression of antigens in relation to progression of disease. The availability of the new approaches to the early diagnosis of systemic candidosis is summarized in Table 3.

5 VALUE OF NEW APPROACHES IN DIAGNOSIS OF SYSTEMIC FUNGAL INFECTION

In clinical practice, there are many frequently asked questions related to the development and application of new methods, these include:

How frequently should sera be examined? How sensitive is the assay in different forms of the disease? How sensitive is the assay at different stages of infection? Is the assay likely to make the diagnosis before other diagnostic procedures? Is sensitivity affected by the underlying condition, e.g., neutropenia, AIDS? Can the assays be used to distinguish between systemic infection and colonization? Can the assays be used prognostically? Can the assays be used to monitor response to treatment and to detect relapse? Is the antigen detected resistant to proteinase treatment, freezing, or thawing? Is the assay species specific?

In relation to aspergillosis and candidosis, very few answers exist; only in the diagnosis of cryptococcosis and the systemic fungal infections seen principally in the Americas is serology the definitive diagnostic test.

Molecular techniques thus far have been shown to be a highly sensitive diagnostic tool for the detection of low amounts of infectious fungi in diverse specimens and points to a high value in clinical use; however, most PCR assays applied in clinical diagnostics still show problems arising from contamination by ubiquitous fungal conidia. Further clinical evaluation in prospective multicenter studies to define the predictive value of these novel assays is at present in progress.

6 CONCLUSIONS

To be successful, pathogenic fungi have to establish themselves in the host. This is achieved in a number of ways, including: an increase in biomass, morphological transition, spread from the original site of colonization or infection, an increase in functional aggressiveness, penetration of barriers, invasion and damage of living tissues. In the process of invading host tissue, there is an increase in fungal-specific products due to normal excretory or secretory mechanisms, natural autolysis, and an attempt by the host to damage or destroy the invading pathogen. These excretory, secretory, or breakdown products could potentially be utilized as specific markers of systemic disease. A few of these areas are being explored.

The diagnosis of systemic fungal infections is realistically based on three elements: host status, clinical features, and mycological findings. Techniques are now available for testing this approach and putting it into clinical practice. The type and timing of treatment depends upon the likelihood of disease. Defining infection allows pre-emptive treatment.

Table 3 Immunoassays for the diagnosis of systemic candidosis

Marker	Method	Availability	
anti-*Candida* immunoglobulin	ELISA	Commercial	Kits
Mannan	ELISA	Commercial	Kits
Glucan	Colorimetric	Commercial	Kits
Glucan	ELISA	Investigational	
Arabinitol	Chromatographic	Investigational	
Arabinitol	Colorimetric	Investigational	
DNA	PCR	Investigational	

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The new generation of immunoassays may afford the most effective and economic management of systemic fungal infection.

The clinical value of serological assays in detecting antigenic structures to diagnose systemic *Candida* infections in patients with malignant hematological disease has been low up to now. Concerning IA, the novel serological tools seem to play a more important role, in spite of limitations due to technical problems and contamination rates. The validation in prospective multicenter studies of defined large patient populations is still lacking.

Serological assays designed to detect IA are more suggestive of invasive disease, but should only be used in combination with clinical features and the finding of imaging techniques. They may at present be a helpful part of the diagnostic procedure in individual febrile neutropenic patients, but they cannot be generally recommended for routine clinical use. The value of molecular diagnostic assays seems to be higher, primarily in the clinically important attempts to improve early diagnosis of IA. The potential of these methods will only be realized in prospective trials. Preliminary results suggest that in future these assays make possible the preemptive treatment of high risk patients or the characterization of a patient population requiring intensification of antifungal treatment, in order to improve further the poor prognosis of patients with these life threatening infections.

Many studies have demonstrated that PCR is an excellent means of correctly identifying particular species, with most studies reporting a sensitivity of 100%. Similar levels of specificity and sensitivity can be attained using conventional diagnostic methods; however, the main advantage of PCR over these methods is the speed with which these results can be obtained. Most culture based methods take a minimum of 48 h (often longer in the case of slower growing species). In contrast PCR tests, even with additional adaptations to improve sensitivity and specificity, can be performed in as little as 8 h. Of course, this speed comes at a cost. Despite more than a decade of research, the necessary equipment is still relatively expensive, and the techniques used involve complicated manipulations that are labor-intensive and are not amenable for use with large numbers of samples. Consequently, the majority of clinical diagnostic laboratories still rely on conventional nonmolecular procedures such as culture and serology.

There is a clear need for the development of improved methods to replace or complement currently used methods to replace or complement currently used methods for the diagnosis of deep-seated fungal infections. The technique that has been investigated most extensively and that offers the greatest potential is PCR. Unfortunately, the current state of PCR technology is not sufficiently advanced to allow its widespread application for routine diagnosis. However, research into adapting PCR so that it is more applicable for use in diagnostic laboratories is continuing, and several developments suggest that rapid, use-friendly PCR-based technology will be applied to high-volume use in the routine diagnostic laboratory in the not too distant future. In this new era of geneomics and microarray technology, it is only a matter of time before techniques involving microchips containing DNA arrays are applied to the automation and miniaturization of molecular methods for the diagnosis of infectious diseases, including invasive fungal infections. For the foreseeable future, routine diagnostic laboratories will have to rely on the conventional culture- and serology-based tests currently in use. However, even if rapid molecular tests are developed for routine diagnostics, it is likely that these will still have to be used in conjunction with nonmolecular methods.

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Candidiasis

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1 INTRODUCTION

The term Candidiasis is used to refer to the diseases caused by fungi of the genus *Candida*, a common saprophyte in the human gastrointestinal tract and elsewhere (Benett 2001; Ellis 1994). Until recently, *Candida* infection was thought to be caused mainly by *Candida albicans*, however, in the last few years other *Candida* species, such as *C. parapsilosis*, *C. tropicalis* and *C. krusei*, have been found to be important causes of disease. These species can be important pathogens in immunodepressed patients. *Candida glabrata* (syn. *Torulopsis glabrata*) causes the same spectrum of diseases (Benett 2001). *Candida* species, particularly *C. albicans*, colonize the human gastrointestinal, respiratory and reproductive tracts, skin, and nails. *C. albicans* is a constituent of the normal oral and gut flora of most healthy people although the carriage rate varies substantially in relation to the methods used to detect it. Carriage is highest in immunodepressed, diabetic or hospitalized patients. Since *Candida* comprises a highly heterogeneous group of yeasts and some species such as *C. parapsilosis* are a mixture of genetically different organisms; different immunopathogenic mechanisms can be involved in infections by different species (Ashman 1998; Ellis 1994; Lehman 1993; Shoham and Levitz 2000).

2 CANDIDA SPECIES

2.1 *Candida* Versus *Torulopsis*

The inclusion of species of *Torulopsis* in the genus *Candida* has been proposed. Originally the genus *Torulopsis* was separated from *Candida* by the absence of pseudomycelium and the lack of hyphae *in vivo* (Ellis 1994; Shoham 2000). Yarrow and Meyer (1998) have however suggested that all *Torulopsis* species should be included in the genus *Candida*.

2.2 *Candida Albicans*

Candida albicans occurs naturally as a commensal of mucous membranes and in the digestive tract of humans and animals. At least 70% of *Candida* strains isolated from sites of infections have been identified as *C. albicans,* and it has been reported as pathogenic in all forms of candidiasis. *C. albicans* can also be isolated from sources contaminated by human or animal excreta (Ellis 1994).

2.3 *Candida* (*Torutopsis*) *Glabrata*

Candida glabrata is found on body surfaces and is often isolated as an incidental finding from the skin, urine and faeces. It is an opportunistic agent in systemic and superficial infections, especially in immunocompromised hosts, and it has been isolated from patients with septicemia, pulmonary infections, endocarditis and pyelonephritis (Edwards 1995; Ellis 1994).

2.4 Other *Candida* Species

Other species of *Candida* that have been implicated in human infections are: *C. (Torulopsis) fumata*, *C. (Torulopsis) haemuloni*, *C. tropicalis*, *C. kefyr pseudotropicalis*), *C. krusei*, *C. (Torulopsis) norvegiensis*, *C. parapsilosis*, *C. viswanathini*, *C. lusitaniae*, *C. guilliermondi*, and

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C. dubliniensis (Edwards 1995; Ellis 1994; Schorling et al. 2000).

3 CANDIDA ANTIGENS

Candida cell walls are a complex of different macromolecular compounds including polysacharides (glucans, mannans, chitin), proteins, lipids, and capsule polysaccharides. At least 78 antigenic fractions can be obtained from the cytoplasm of *C. albicans* and metabolic antigens obtained from dialyzed culture fluids containing mannans and glucans. The cell wall is an insoluble glucan–protein complex and contains two soluble glucamanan protein complexes with antigenic properties dependant on their mannose/nitrogen contents (Kaufman and Standard 1987). Various cell wall and somatic antigens from *Candida* species are able to induce an immune response including wall mannan glycoproteins, cytoplasmic somatic protein, indefined heat labile glycoprotein, antigen D-arakinitol, D-mannase, etc. (Cutler 1998). These antigens are very complex, and infected, allergic, or immunocompromised patients often respond only to certain cell wall, cell membrane, or cytoplasmic components. In general, the response in deep infections is mainly directed towards cytoplasmic components, and in superficial infections or allergy it is generally directed towards cell membrane or cell wall components (Lehman 1993). The major cell surface antigen of *Candida* spp. is a mannan component of the cell wall. *C. albicans* isolates have been serotyped into two groups (A and B) based on differences within mannans. Cytoplasmic antigens have also been extensively studied (Lehman 1993; Shoham and Levitz 2000), and humoral immunity to a cytoplasmic antigen has been reported in systemic candidiasis (Lehman 1993; Shoham and Levitz 2000). Testing for the presence of cytoplasmic antigens directly in serum can now be performed by PCR (Bryant 1992; Cook 1996).

3.1 *Candida Albicans* Serotypes

Three different *C. albicans* serotypes have been identified (A, B, C), and these differ by serological cross-reactions of mannans from different strains. The main antigenic determinant of the A serotype is the Mannoheptaose in the lateral chain, whereas serotype B has a mannohexose in this position (Cutler 1998; Lehman 1993; Shoham and Levitz 2000).

3.2 Antigenic Cross-reactivity

Antigens from *C. albicans* and other *Candida* varieties have strong cross-reactivity. *Candida* antigens can cross-react with other yeasts including *Saccharomyces cerevisiae*, *S. minor*, and *Pityrosporum orbiculare*, the last of which causes certain clinical forms of allergy (Huang et al. 1995; Koivikko et al. 1998; Savolainen et al. 1998).

4 CANDIDA ANTIBODIES

Antibodies to *C. albicans* can be either IgG, IgA, IgM, or IgE. Antibodies can be raised against mannans or somatic antigens. IgA antibodies have been found in saliva and vaginal secretions. Most people have low levels of antibodies when compared to *Candida* mannans. In infected patients, these levels will be higher and, IgG, and after early infection, IgM are usually found (Mathews and Burnie 1998). Patients with allergic or infectious diseases or the hyper IgE syndrome can present elevated levels of specific IgE antibodies. However, in most cases specific IgE is negative even in the presence of clinical disease and positive skin tests, probably because the available systems are directed against only a fraction of the candidal antigenic mosaic (Ashman 1998; Lehman 1993; Savolainen et al. 1998; Shoham and Levitz 2000).

5 HUMAN DISEASES DUE TO CANDIDA

Candida species can cause different forms of human diseases including allergy, cutaneous and mucoral candidiasis, organ related infection, systemic diseases, and endocrine syndromes.

5.1 Allergy

Candida species can cause allergic rhinitis, sinusitis, asthma, combined allergic rhinitis asthma syndrome (CARAS), hypersensitivity pneumonitis, allergic broncho-pulmonary candidiasis urticaria or urticaria angioedema syndrome, and atopic eczema, and these have been recognized for many years (Dhivert 1988; Girard and Gumovski 1985; Koivikko et al. 1998; Savolainen et al. 1993; 1998; Sclafer and Thibault 1986). Urticaria seems to be the most common form of *Candida* allergy (Sayag 1992), and there is substantial literature suggesting that subtle infection with *C. albicans* may be a cause of chronic urticaria. In a large study of over 200 patients with chronic urticaria, almost 20 patients gave an immediate positive reaction to skin testing with an extract of *C. albicans.* These patients recovered from the urticaria when placed on antifungal drugs and a low yeast diet. However, the results of skin testing are not always predictive and a patient with negative skin tests can be treated with the same therapy (James and Warin 1971). In some cases a diet without yeasts (bread, beer, cheese, wines) but with rice, lamb, and water may identify some patients allergic to *Candida* or with cross reactions to other yeasts (Sclafer and Thibault 1986). In a survey of mucocutaneous candidiasis 12 out of 58 patients had urticaria. In a group of 145 patients (103 females and 42 males) with urticaria and angioedema, prick skin tests were

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positive in 45 (31%) of the patients and intradermal tests in 140 (97%). *C. albicans* has been also implicated in atopic dermatitis (Savolainen et al. 1993). Asthma and allergic rhinitis due to allergy to *Candida* spp. have been reported by several workers (Dhivert 1988; Girard and Gumovski 1985; Savolainen et al. 1998; Sclafer and Thibault 1986). Asthma, allergic rhinitis, or combined allergic rhinitis asthma syndrome are difficult to diagnose due to nonspecific clinical history, the limited value of prick skin tests, and the fact that specific IgE is seldom found due to the low sensitivity of available methods.

5.2 Candidiasis

In practice candidiasis consists of a wide range of both systemic and localized diseases. Disseminated candidiasis is more common in immunocompromised hosts either due to iatrogenic agents (immunosuppression) or infections, including AIDS.

5.2.1 Oral Candidiasis

Oral candidiasis is one of the most common forms of candidiasis. Oral or oropharyngeal candidiasis includes thrush, glossitis, stomatitis, and angular cheilitis (perlèche). Acute oropharyngeal candidiasis is rarely seen in healthy adults, but may occur in up to 5% of newborn infants and 10% in the elderly (Ellis 1994). However, the use of broad spectrum antibodies, corticosteroids, immunosupressive drugs, and radiation therapy can trigger oral candidiasis. In practice the condition is often associated with HIV infection and also with malignancies, leukemia, lymphomas, neutropenia, and diabetes mellitus (Appleton 2000; Ashman 1998; Benett 2001; Ellis 1994; Lehner 1993). Lehner (1993) described four types of oral candidiasis. (a) Acute pseudomembranous candidiasis, usually called thrush, is observed in infants and debilitated adults namely with leukemia, lymphoma, or diabetes. Drugs are important predisposing factors, particularly broad spectrums antibiotics, corticosteroids, or imrnunosupressives. Local antibiotics or corticosteroids can also enhance candida infections. Clinical manifestations are usually symptomless with papules or cotton–wool exudates which can leave a redish mucosa. (b) Acute atrophic candidiasis—this form may follow acute pseudomembranous candidiasis, which is associated with broad spectrum antibiotic sore-tongue. It is usually painful with a smooth erythematous tongue with cheilitis (perléche) and less often, inflammed lips and cheeks. Symptoms can include burning or dryness of the mouth, loss of taste, and pain on swallowing. (c) Chronic atrophic candidiasis is usually known as denture stomatitis and produces diffuse erythema of the palate, limited to the denture bearing mucosa. The lesion is usually symptomless but is often associated with angular cheilitis. (d) Chronic hyperplastic candidiasis is a rarer form of oral candidiasis presenting as a firm white patch, which cannot be rubbed off and generally affects the tongue, cheek, and lips. This is also called Candidial leukoplakia (Lehner 1993). This lesion is very persistent and usually resistant to treatment.

5.2.2 Mucocutaneous Candidiasis

Mucocutaneous candidiasis is very common. Some forms are congenital, others acquired, and the more severe forms are often linked to auto-immune diseases or immunodepression. The chronic form has a particular presentation and is usually considered as a primary immunodeficiency (I.U.I.S. Scientific Committee 1999; Palma-Carlos and Palma-Carlos 1991). Chronic mucocutaneous candidiasis should be viewed as a spectrum of disorders in which the patients have persistent and/or recurrent candidiasis of the skin, nails, and mucous membranes (Kirkpatrick 2001). Some cases of muco-Candidiasis have a genetic predisposition, and the suggested immunological abnormality is a failure of the patient's T lymphocytes to produce the cytokines that would induce cell-mediated immunity to *Candida* (Kirkpatrick 1989; 2001). Defects in phagocytosis, leukocyte function, or T-cell mediated immunity have also been reported (Appleton 2000; Ashman 1998; Palma-Carlos and Palma-Carlos 2001), and endocrine disorders such as hypoparathyroidism, addison failure, hypothyroidism, diabetes mellitus, disfunction of the thyroid, and polyglandular autoimmune disease have also been described. In general, the patients affected are mainly children (Appleton 2003; Benett 2001). In addition to the chronic form, there are other mucocutaneous forms, (a) Chronic localized mucocutaneous candidiasis—starts in childhood as an intractable infection of the nails and sometimes the adjacent skin of hands and feet. The infection can extend to other skin sites. (b) Chronic localized mucocutaneous candidiasis with granuloma—this condition starts in infancy and is clinically similar to previous one, with the addition of granulomatous lesions affecting the face and scalp. About 25% of infected children also have recurrent respiratory tract infections (Lehner 1993). (c) Chronic localized mucocutaneous candidiasis with endocrine disorders—this is usually found in children or young adults, and there is a strong familial incidence. Endocrine abnormalities are generally preceded by candidiasis. Hypoparathyroidism, hypothyroidism, or Addison's disease are the most common among these, and clinical features are similar to the localized muco-cutaneous variety.

Other forms include intertrigo, paronychia, and onycomycosis often associated with oral, oesophageal, and vulvovaginal candidiasis. Pulmonary extensions from the mouth can also be seen in more severe cases. (Ellis 1994; Lehner 1993). Intertrigo due to *Candida* is more common in axillae, groin, inter, and sub-mammary folds, intergluteal folds, interdigital spaces, and umbilicus. Moisture, heat, friction, and maceration of the skin are the most important triggering factors, and obesity, diabetes, and the use of broad-spectrum antibodies are additional factors. This form of candidiasis presents as a macular moist erythematous or rash with satellite lesions on the surrounding healthy skin. Diaper

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candidiasis is common in infants where there is prolonged moisture and local skin maceration associated with irritation due to dirty diapers. The erythematous lesions are accompanied by satellite pustules mainly localized in skin folds and creases. Apart from of cytostatics, other iatrogenic factors are corticosteroids and broad spectrum antibiotics (Appleton 2000; Ashman 1998; Ellis 1994; Lehner 1993).

5.2.3 Onycomycosis

Paronychia of the finger nails can affect subjects, who's fingers have been continually immersed, especially in sugar solutions or detergents that macerate the nail folds and cuticle. The candidiasis lesions are painful erythematous swellings around the affected nail. The chronic infection may lead to onychomycosis with total detachment of the cuticle from the nail plate. Chronic *Candida* onychomycosis often causes complete destruction of the nails and is seen in patients with chronic mucocutaneous candidiasis or associated diseases such as diabetes, hypoparathyroidism, hypothyroidism, Addison's disease, diabetes mellitus, malignancies, or immunosuppression (Benett 2001; Ellis 1994; Lehner 1993).

5.2.4 Vulvovaginal Candidiasis

Vulvovaginal candidiasis is a common condition in women, and is often associated with the use of broad spectrum antibiotics. The final months of pregnancy, *diabetes mellitus*, and local conditions such as low vaginal pH, oral contraception, and sexual activity may contribute to the vulvovaginal infection. The infection can spread from the genitals and extend to the perineum and the inguinal area. Symptoms are intense vulvar pruritus, burning erythema, and dyspareunia associated with a creamy white curd-like discharge. Refractory vaginal candidiasis may be a later presentation of an attenuated form of chronic mucocutaneous candidiasis or of HIV infection, hypoparathyroidism or *diabetes mellitus* and may be due to either *C. albicans* or other *Candida* species (Benett 2001; Ellis 1994; Moraes 1993; Sobel et al. 1998).

Vulvovaginal candidiasis is often recurrent, and severe vulvovaginal candidiasis is by far the most common mucosal fungal infection in women (Nyirjesry 2001; Shoham and Levitz 2000). Individuals with deficient cellular immunity or metabolic diseases may have higher rates of infection. Vaginal immunity shows a high level of independence from systemic immunity and 0.5 to 10% of females without immune deficient cellular immunity can present complicated *Candida* vaginitis. Innate and acquired immunity tends to maintain a balance between vaginal immune response and *Candida* colonization (Shoham and Levitz 2001). Local defenses, aside of innate immunity, consist of IgA and IgG antibodies. Production of cytokines by vaginal epithelium can also play a role in defense (Monteiro et al. 1996; Moraes 1993; Moraes and Taketomi 2000). Vulvovaginal candidiasis may be classified into complicated and uncomplicated forms (Sobel et al. 1998). In uncomplicated forms, the severity is mild to moderate, the frequency sporadic, the host is normal, and the infecting organism is *C. albicans*. In complicated forms the disease is severe and recurrent, and the host is abnormal, being either immunocompromised (primary or acquired immunodeficiency) or presenting an endocrine disease. Patients presenting one of the features reported-severity, recurrence, metabolic or immune disease or non-*albicans* species of *Candida* are defined as complicated vulvovaginitis. Complicated vulvovaginal candidiasis is associated in most cases with candidiasis at other locations, including mouth, skin, or nails. Uncomplicated vaginitis is seen in 90% of patients and usually responds readily to short-course oral or topical treatment. The complicated form of vulvovaginitis is seen in the remaining 10% of patients, generally as recurrent bouts of infection after local and systemic treatment (Sobel et al. 1998). In our research, 46 out of 57 (80%) females with mucocutaneous candidiasis patients presented complicated vulvovaginitis. The majority of these patients also had other candidiasis conditions either as skin infection (57%), onycomycosis (24%), or oral thrush (67%). Different patterns of defective immune response have been reported. (1) 66% of patients had a decrease in cytotoxic cells, most often natural killer (NK) cells, but also in CD8 cytotoxic cells. (2) In 5% of patients NK and CD8 cells were normal but IgG specific antibodies to *Candida* were absent. Globally a defect in the defense factors implied in antibody cellular cytotoxicity (ADCC) NK or CD8 cells, or *Candida* antibodies has been found in (80%) of patients studied. These data suggest that combined cellular and humoral defects in antibody dependent cellular cytotoxicity (ADCC) play a major role in most cases of complicated candidial vulvovaginitis.

5.2.5 Candidial Balanitis

Candidial balanitis is much more rarer than the female counterpart. In the case of balanitis the partner should be investigated for vulvovaginitis and immunodeficiency, and endocrine disease or *diabetes mellitus* excluded (Ellis 1994). Infections are more commonly seen in uncircumcised men and poor hygiene may contribute. The symptoms include erythema, pruritus, and vesiculopustules on the penis or prepuce (Benett 2001; Ellis 1994).

5.2.6 Oesophageal Candidiasis

Candida albicans is the most common species isolated in oesophageal candidiasis even among immunocompromised hosts. However in some cases *C. glabrata* and *C. krusei* have been implicated. Oesophageal candidiasis is frequently associated with AIDS or immunosuppresion following cytostatics and oral candidiasis is generally associated. Symptoms are a burning pain on the retroesternal area, dysphagia, nausea, and vomiting. Endoscopy shows white mucosal plaques with erythema resembling oral candidiasis. These symptoms may lead to a reduction in food and liquid intake and may significantly reduce the quality of life. Viral

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infections with herpes simplex virus (HSV) or cytomegalovirus (CMV) may also be associated (Benett 2001; Ellis 1994; Rex et al. 1998).

5.2.7 Gastrointestinal Candidiasis

Gastrointestinal candidiasis can be associated with hematological malignancies, often presenting as ulcerations of the stomach, and less frequently of the duodenum and intestine leading to peritonitis and disseminated candidiasis. Invasion of the digestive tract is often followed by the excretion of large numbers of *Candida* cells which can be found in the stools in much larger numbers than when *Candida* is a commensal in nonimmunocompromised hosts (Benett 2001; Ellis 1994).

5.2.8 Hepatic and Hepatosplenic Candidiasis

Hepatosplenic candidiasis generally corresponds to a chronic disseminated candidiasis and occurs mainly in immunocompromised patients or those with severe neutropenia, usually acute leukemias. Clinical presentation is usually a fever, hepatosplenomegaly and increased blood alkaline phophatase. Abcesses containing small number of pseudohyphae can be found, but blood and biopsy cultures are usually negative (Benett 2001; Ellis 1994; Kontoyiannis et al. 2000).

5.2.9 Candidial Infections of the Gallbladder, Pancreas, and Peritoneum

Infections of the gall bladder and pancreas are rare but can create additional complications in immunocompromised patients. Peritoneal candidiasis can be related to peritoneal dialysis catheters, and catheter removal is often required for the successful treatment of the infection. Candidial peritonitis can also occur due to gastrointestinal perforation by ulcers, diverticular colitis, and surgical or trauma injury to the gut wall. In these cases *Candida* is usually part of a polymicrobial infection. The symptoms are fever, abdominal pain, tenderness, and a cloudy peritoneal dialysate with high leukocyte count. Candidial peritonitis can disseminate in severely immunocompromised patients (Ellis 1994; Kontoyiannis et al. 2000).

5.2.10 Urinary Tract Candidiasis

Risk factors for candidial urinary tract infection include urinary tract catheters, recent antibiotic therapy, and advanced age. *Candida* is now the most frequently isolated organism from the urine of patients in surgical intensive care units (Benett 2001; Shoham and Levitz 2000). However, in most patients isolation of *Candida* represents only colonization and is a benign event. *Candida* excretion could be transient and asymptomatic as a result of antibiotic or corticosteroid treatment promoting the growth of *Candida* in the gastrointestinal and genital tracts. Most lower urinary tract infections result from a local spread of *Candida*. It is difficult to distinguish between colonization, contamination, and infection, but it is important to distinguish between a renal infection and simply a cystic infection (Benett 2001; Ellis 1994).

5.2.11 Candidial Endocarditis, Myocarditis, and Pericarditis

Endocarditis is the most common form of cardiac candidiasis. These infections have high morbidity and mortality. Preexisting valvular disease with concomitant intravenous catheters and antibiotic treatment, intravenous drug abuse, heart surgery, and valve problems are all predisposing factors. Clinical symptoms include fever, cardiac murmurs, congestive heart failure, anemia, and splenomegaly. Myocardial abcesses, arterial emboli, and purulent pericarditis can occur as further complications of *Candida* septicemia or surgery (Benett 2001; Ellis 1994).

5.2.12 Candidal Supurative Phlebitis

A localized phlebitis that may also be due to *Candida* infection can occur in immnocompromised patients. These patients have had either a central vein or a peripheral vein linked to local catheter (Benett 2001).

5.2.13 Pulmonary Candidiasis

Pulmonary candidiasis leading to pneumonia can appear in 2 forms. (1) Bronchial extension or the aspiration of *Candida* from oropharyngeal material, this rarely results in primary *Candida* pneumonia. (2) More commonly, a hematogenous dissemination can cause a diffuse pneumonia that generally along involves multiple other organs. Pulmonary candidiasis is difficult to diagnose, and definite diagnosis requires either a culture or histopathological confirmation. The presence of yeasts in alveolar lavage or sputum specimens is not specific. Benign colonization of the airways with *Candida* or contamination of the respiratory secretions with oropharyngeal material is much more common than either of the two forms of true *Candida* pneumonia (Benett 2001; Ellis 1994).

5.2.14 Osteoarticular Candidiasis

Arthritis may occur in conjunction with disseminated candidemia in neutropenic patients or neonates. Prostitic or rheumatoid joints are also susceptible, by either hematogenous dissemination, direct inoculation during surgery or intra articular corticosteroid injection. The knee is the main site involved resulting in pain on weight bearing on full extension. Candidial osteomylitis can also be observed (Benett 2000, Ellis 1994).

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5.2.15 Candidial Meningitis

Candidial meningitis is a rare condition seen predominantly in low birth weight neonates, in septicemia and in patients with hematological malignancies, complicated neurosurgery or intracerebral prosthetic devices. Symptoms are fever and meningeal irritation (Benett 2001; Ellis 1994).

5.2.16 Candidal Endophtalmitis

Candidal endophtalmitis is often associated with candidemia, catheter, or drug abuse. Lesions are often localized near the macula and the patients complain of cloudy vision. Chorioretinitis is a common form of presentation (Appleton 2000; Ellis 1994).

5.2.17 Neonatal and Congenital Candidiasis

Low birth weight and age, prolonged intravascular catheterization, and the use of broad spectrum antibiotics are the main conditions which can cause systemic candidiasis in neonates. Congenital candidiasis acquired *in utero* is usually confined to the skin in the form of a generalized erythematous vesicular rash (Ellis 1994).

5.2.18 *Candida* Septicemia and Disseminated Candidiasis

Candida septicemia is also called candidemia and defined as the presence of yeasts in the blood with or without visceral involvment. Candidal dissemination by the blood stream can lead to colonization of one or more organs and the formation of multiple microabcesses. *Candida* septicemia is nowadays a frequent cause of sepsis in hospitalized patients. This situation is facilitated by the prolonged or repeated use of intravenous catheters, use of broad-spectrum antibiotics, urinary catheters, surgical procedures, immunosuppressive drugs, corticosteroids, parental nutrition, severe burns, or impairement of the mucosae. Resistant fever in neutropenic patients with tachycardia and dyspnea often accompanied by hypertension and skin lesions suggests candidemia (Benett 2001; Ellis 1994; Klepser et al. 1998; Lewis and Klepser 1999; Shoham and Levitz 2000; Wey et al. 1998).

5.3 Association with Auto-Immune Endrocrine Syndrome

Candidiasis can be associated with autoimmune endocrine syndromes affecting different endocrine glands. The association of chronic mucocutaneous candidiasis, hyperparathyroidism and Addison's disease is usually found in auto-immune polyendocrine syndrome type I (APS-I) or poly-glandular autoimmune (PGA-I). Other associations with endocrine diseases have also been reported, namely with autoimmune thyroid disease in the group of PGA II or on partial PGA-Syndrome (Palma-Carlos and Palma-Carlos 2001; Song et al. 1998).

6 GENERAL METHODS FOR CANDIDA ANTIBODIES AND ANTIGEN DETECTION

Different classes of antibodies directed to *Candida* have been reported. IgG, IgM, and IgA *Candida* specific antibodies can be detected in Candidial infections, whereas IgE specific antibodies are found in same cases of allergic reaction to *Candida* antigens. Functionally *Candida* antibodies can be agglutinating, precipitating, or complement binding (Bryant 1992; Cook 1996; Lehner et al. 1990; Savolainen et al. 1998). Detection can be by fluorescent methods, ELISA or serological titration (Lehman 1993; Lehner et al. 1990). *Candida* specific IgG3 antibodies have been reported in asthma (Girard and Gumovski 1985) and IgE antibodies in *Candida* allergy. Specific *Candida* IgE antibodies can be directed against proteins or polysaccharide antigens (Matthew 1988, Savolainen et al. 1998).

6.1 Immunodiffusion and Counter Immunoelectrophoresis

Immunodiffusion techniques can reliably detect antibodies in candidiasis even in immunocompromised hosts. A heat-stable cytoplasmic antigen is used with positive control serum containing at least three of the seven known precipitins. Formation of one or more bands between the reagent antigen and the patients' serum is considered a positive reaction. Candidiasis is presumptively identified when serial specimens taken at different times shows an increasing number of precipitin bands or become positive. Counter immunoelectrophoresis gives comparable results (Bryant 1992; Cook 1996).

6.2 Latex Agglutination Test

The latex agglutination test is semiquantitative and can be useful both for diagnosis and as a prognostic marker (Bryant 1992).

6.3 Indirect Immunofluorescence

Indirect immunofluorescence has been accepted in the last few years as a reliable method for detecting *Candida* IgG and IgM antibodies. An IgG titer higher than 320 suggests a recent infection. An increase in IgG titer or a positive IgM antibody suggests a systemic infection. IgG is usually detected in normal or slight increased titers by this method in cases of severe mucocutaneous candidiasis (Lehner 1990).

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6.4 Antigen Detection

A double antibody sandwich enzyme immuno assay can be employed to detect *Candida* antigens. Concentrations greater than 2 μg/ml are presumptive evidence of active candidiasis (Bryant 1992; Cook 1996).

7 CELLULAR IMMUNITY IN CANDIDIASIS

Cellular immunity can be studied by *in vivo* or *in vitro* methods. The *in vivo* technique is a delayed hypersensitivity skin test to *Candida* antigens which is generally considered to be negative in most of the candidiasis patients (Lehman 1993; Palma-Carlos and Palma-Carlos 2001). Studies of cell populations in candidiasis are nowadays by choice performed by flow cytometry to detect T, B, and non T, non B cell populations. In the last few years it has been suggested that NK cells play a role in defense against *Candida* (Guduri and Ballas 2002; Palma-Carlos and Palma-Carlos 2001; Segura and Ballas 1998), due to either of their lack of cytotoxicity or as a major source of interferon gamma. A decrease in NK function has been reported (Guduri and Ballas 2002; Segura and Ballas 1998). A recent study showed that T-cells marked by CD2 were in the normal range for 44 HIV negative patients. CD8 cytotoxic cells were in the normal range in 36 patients (92%) and decreased in 6 (8%). The more relevant aspect of these studies was the natural killer cells (Palma-Carlos and Palma-Carlos 2001). IgG receptor CD16 and NK marker CD56 have been evaluated in Pan T negative CD3 cells. Both NK markers were studied in 51 patients. The CD16 count was normal in 46 and decreased in 4 patients (20%). The CD16 numbers (as percent of lymphocytes) were decreased in 13 patients (23%) and normal in 38. The CD56 count was decreased in 7 patients (13%) and normal in 16 (31%). The percentage of CD56 was decreased in 15 patients (29%) and normal in 36. About 66% of the patients with mucocutaneous candidiasis and complicated vulvovaginitis had a cellular immunity defect. In 5% of the patients, cellular immunity was normal but antibodies implied in ADCC were absent. These two different patterns suggest an important ADCC mechanism in the defense against *Candida* infections. NK cells and antibodies were involved, probably through NK CD16 receptors for specific IgG antibodies directed to *Candida.* Delayed type hypersensitivity to *Candida* with systemic or organ related symptoms could be detected *in vitro* by flux cytometry. The increase in activation markers is also found after cell culture in the presence of *Candida* antigens (Brunet et al. 2001).

8 IMMNOPATHOGENESIS OF CANDIDIASIS

Candidiasis, in its different forms, occurs when local or systemic defenses are defective, as innate immunity is a dominant protective mechanism against candidiasis. Fungi have been implicated as a cause of immunosuppression in humans and animals. The administration of killed *C. albicans* to mice results in transient depression of T-lymphocyte dependent mitogen responses (Ashman 1998), and human chronic mucocutaneous candidiasis can be associated with depressed delayed-type hypersensitivity responses (Appleton 2000). Neutrophils and monocytes can damage and kill yeast cells, hyphae, and pseudophyphae but the large size of hyphae and pseudo hyphae does not allow phagocytosis. In such cases several phagocytes may be associated in extra cellular killing (Shoham and Levitz 2000). Cell mediated immunity including NK cells play a dominant role in the control of candidiasis as shown in patients with cell mediated immunity defects (Ashman 1998; Lehman 1993; Shoham and Levitz 2000).

Multiple defects on cell mediated immunity have been described in candidiasis but the consequences are not clear. In mucosal candidiasis, cellular immunity may be a dominant factor. At the cellular level, the development of TH1 cellular mediated immunity responses are associated with protection against invasive disease. This is in contrast to TH2 responses, which are nonprotective. The network of THI and TH2 cytokines, the timing of their release, and their relative concentrations play a role in this (Appleton 2000; Ashman 1998; Shoham and Levitz 2000). The cellular immune response can tolerate a small amount of *Candida* in mucosae and *Candida* can have an immunosupressive effect (Rivas and Rogers 1983). The relative order of activation of TH1 and TH2 cytokines as well as the stimulation of NK cells must play a role in cell response to *Candida*. Antibodies are also implicated in host response to *Candida*. IgG, IgM, and antibodies can be found in most patients but are probably ineffective in the absence of cytotoxic cells. Individual cells not fundamentally immune as platelets and endothelial or epithelial cells can also play a role in the defense against *Candida* (Appleton 2000; Ashman 1998). IgE specific antibodies are seldom positive for *Candida* infections, probably due to technical limitations in their detection. Immunoblot assays performed on patients with disseminated candidiasis suggest that higher levels of sustained IgG antibodies can be associated with an improved outcome. However, the significance of antibodies in candidiasis remains controversial. Patients with predominant antibody defects do not appear to suffer from increased rates of candidiasis. It has been suggested that there are protective, nonprotective, and indifferent antibodies for *C. albicans*. It is possible that the contribution of antibodies to the defense against candidiasis depends on the relative proportion of the different classes of antibodies in the serum or mucosae. The cooperation between antibodies and cells in ADCC however probably plays a major role. The cells involved in defense, mainly NK cells, must express high affinity receptors for IgG (circular dichroism 16) and kill *Candida* only after binding antibodies. Therefore *Candida* immunity can be disturbed either by an IgG antibody defect or by a decrease in the number or the activity of NK or CDS cytotoxic cells (Palma-Carlos and Palma-Carlos 2001). The complement system can

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also be involved, and following incubation with normal serum *C. albicans* activates complement through both the classic and alternative pathways. Immunoglobulins directed against *Candida* mannan antigens activate the classic pathway. However, perhaps, because of their thick walls, *Candida* and other fungi are generally resistant to complement mediated lysis and are not killed by serum (Kozel 1998). The predisposing factors to fungal infections in humans are diverse, local, or systemic, comprising impaired teguments, neutropenia, chronic granulommatous disease, impaired cell mediated immunity or ineffective antibody dependent cytoxicity. These different conditions can coexist in the same patients and can be increased by diagnostic or therapeutic manipulations. Defense mechanisms in candidiasis are clearly heterogeneous and different abnormalities may each play a role in clinical presentation (Appleton 2000; Benett 2001; Kirkpatrick 2001).

9 THERAPY OF CANDIDIASIS

9.1 Prevention and Therapy of Candidiasis

Prevention to candidial infection is an important part of the control of candidiasis. Prophylactic strategies are mandatory if the risk of disease is sharply elevated. Patients under therapy that can causes prolonged neutropenia, such as bone-marrow recipients or solid-organ transplants, have a high risk of systemic candidiasis and warrant systematic prophylaxis (Rex et al. 2000; Sobel 2000). Treatment of candidiasis must be considered for both invasive and mucocutaneous candidiasis. The pharmacotherapy of candidiasis is dominated by fluocytosine, amphotericin B, azoles, and the recent echinocandins (Groll and Walsh 2001; Singh 2001; Sobel 2000). General preventive measures such as the removal of catheters and prophylaxic treatment in patients involved in therapy that produces prolonged neutropenia such as bone marrow or solid organ transplant recipients are recommended (Klepser et al. 1998). For mucocutaneous candidiasis, the most current therapy is done with azole anti-fungal agents or with terbinafine for onycomicosis. For some 40 years Amphotericine B has been the sole or the most widely used anti-fimgal agent. The serious side effects associated with amphotericine B have however limited this therapy. Efforts to decrease the toxicity of amphotericine B therapy have lead to lipid-based formulations that have less nephrotoxicity than systemic amphotericine B. Encapsulated amphotericine B with a lipid layer has a minimized toxicity while the activity of the drug is maintained. The subsequent introduction of azole antifungals and Terbinafine has allowed the treatment of candidial infections with much less toxicity. Flucytosine is currently not used in mucocutaneous forms of candidiasis but has some clear advantages over azol compounds, while Terbinafine can be used in onycomycosis as an alternative to fluconazol or itroconazol (Appleton 2000; Rex et al. 2000; Sobel 2000; Sobel et al. 1998). The changing epidemiology of fungal infections and the increase of anticandidial resistance are both serious problems that we will have to face in the next few years (Lewis and Klepser 1999). Epidemiology is dominated by the associated HIV infections and by the use of immunosupressive drugs in oncologic patients or in bone marrow or solid organs transplants. Antifungal resistance can be primary (innate) or secondary (acquired). Innate resistance can be present without antifungal therapy and is often intrusive to certain *Candida* species, for instance resistance to fluconazole in *C. krusei* and to amphotericine B and flucytosine in *C. hitaniue* species. On the other hand repeated exposure of a *Candida* population to an antifungal may select resistant strains or allow the replacement of susceptible variants by intrusively resistant species (Lewis and Klepser 1999; Rex et al. 2000). Acquired secondary resistance occurs during antifungal therapy. The development of resistance to Amphotericine B is rare but resistance to flucytosin is quite common if this drug is used alone. The most common secondary resistance occurs with fluconazol therapy. This resistance is particularly common in HIV infected patients with oropharyngeal candidiasis after prolonged or repeated courses of the drug. However the level of antifungal resistance will probably not increase as rapidly as has been the case for antibiotic resistance in bacteria, as there is no evidence for horizontal gene transfer in *Candida*. Many *Candida* species lack a haploid growth stage and the frequency of mutations causing antifungal resistance is quite low. The spread of antifungal resistance among *Candida* species is more dependent on drug pressure to unmask suppressed resistance genes (Lewis and Klepser 1999) and a well balanced use of antifungals is probably the best defense against candidial resistance. Susceptibly testing is a key point in the treatment of candidial infections. Consistent efforts to improve antifungal therapy have lead to a standardized methodology for yeast susceptibility, detailed in NCCLS M.27 (Rex et al. 2000). Fluconazol and itroconazol are nowadays the most widely used antifungal drugs. Itroconazol usage has been limited by its poor oral availability and lack of a parenteral product. Itroconazol has recently been reformulated and a new more effective oral solution is now available. Furthermore, an intravenous formulation of itroconazole is also being developed. Voriconazole is a new broad-spectrum trinazol antifungal with excellent activity against *Candida* spp. Other broad-spectrum azoles are being developed, such as SOH 56592 and BMS 207147 with expanded spectrum of activity (Rex et al. 2000). Echinocandins are perhaps the antifungals with the greatest potential in candidiasis therapy. Echinocandins act by inhibiting glucan synthesis by the inhibition of 1.3.b glucansynthase, and they show fungicidal activity against a broad range of fungal pathogens including *Candida*. Adverse effects were uncommon in patients receiving MIX-0991, one of the first echinocandins available (Groll and Walsh 1998; 2001; Hoang 2001; Keating and Jarvis 2001).

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9.2 *Candida* Immunotherapy

Candida immunotherapy is not currently recommended due to the lack of double blind placebo controlled assays. However, *Candida* immunotherapy can be tried in some patients when clinical data, skin tests, or antibody are positive (Dhivert 1988). *Candida* immunotherapy has also been tried in mucocutaneous candidiasis mainly with female patients with complicated forms of vulvovaginitis. The results of Moraes (1993); Rigg et al. (1990); Rosedale and Brown (1979); Monteiro (1996); Moraes and Taketomi (2000); Moraes et al. (2000) and our own laboratory all confirm the effectiveness of *Candida* immunotherapy in mucocutaneous candidiasis with 80% success in clinical trials and a correction of NK abnormalities in 70% of the patients. Other trials of immunotherapy have also been carried out, and De Sousa et al. (1976) proposed the treatment of candidiasis with transfer factor. More recently, the American Society of Clinical Immunology (1994) has recommended granulocyte colony stimulating factor (G-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF) as a prophylactic measure in adults or children with febrile neutropenia and high probability of *Candida* infections, as prolonged neutropenia is a risk factor for disseminated Candidemia.

10 CONCLUSIONS

Candidiasis is a rapidly growing subject in internal Medicine, clinical immunology and epidemiology. *Candida* spp. can no longer be considered to be a minor yeast contaminant, and has been recognized as a major cause of human disease, that is not confined to immunocompromised hosts. The emergence of different *Candida* species as pathogens and their differing sensitivity to antifungal drugs places *Candida* in the front line of pathogenic fungi. Despite the growing importance of *Candida* spp. in pathology, knowledge of the defense mechanisms against candidial infection is still incomplete. *Candida* antigens are well known, but the role of humoral and cellular immune reactions in defense against *Candida* has not been completely elucidated. An ADCC mechanism has not previously been postulated, but is probably dominant in most cases. This mechanism can be due either to a decrease in the number or activity of NK cells or CD8 cytotoxic cells, or to the absence of specific antibodies to *Candida*. *Candida* hypersensitivity has been considered for many years as an important factor in allergic diseases. However, in the last few years this importance has decreased. *Candida* allergy cannot be confirmed in most cases due to the lack of specificity of currently available reagents for specific IgE, and the lack of good standardized extracts for *in vivo* tests. This is due to the complexity of *Candida* antigenic mosaic and improvements in methodology are urgently needed. *Candida* spp. have been implicated in a large number of human diseases from immunodeficiencies to speticemia and from allergy or recurrent infections. Diagnosis of candidial diseases or allergy has improved in the last few years, but the underlying mechanisms are far from homogeneous and not completely clarified. Anti-*Candida* therapy is still currently based on older antifungal drugs, and new, more effective drugs free of serious side effects are urgently needed for the newly emerging candidial infections. *Candida* immunotherapy can be employed in certain cases of allergy, and also in mucocutaneous candidiasis with complicated vulvovaginitis where all current antifungal treatments fail. *Candida* immunotherapy can be effective in most cases of *Candida* disease, but standardized extracts and vaccines must be available to assure better diagnosis and immunotherapy. Autoimmunity has been known to be associated with candidiasis for a many years. In chronic mucocutaneous candidiasis parathyroid failure is the most frequently autoimmune disease reported, and in adult patients with mucocutaneous candidiasis, thyroid involvement is common. The subtle mechanism that simultaneously triggers yeast infections and auto-immunity, mimicry or others has not yet been determined.

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Immunizations Against Fungal Diseases in Man and Animals

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1 INTRODUCTION

Since, the time of Pasteur and early microbiology, infectious diseases have traditionally been countered by prophylactic measures entailing vaccination with killed or attenuated microorganisms or their products. Considering that, of communicable diseases with established etiology, fungal diseases were among the first to be recognized, it is rather surprising that the development of vaccines to manage mycoses lags far behind that of bacterial or viral vaccines. This, despite the continuous increase in serious fungal infections in compromised hosts, and in view that introduction of new antifungal drugs still do not completely solve the management of systemic mycoses. Hence, the need for prevention of mycoses by vaccination or boosting of the immune system as adjunct to specific antifungal therapy in order to combat the infections became even more pronounced. The difficulty in the development of antifungal vaccines may reside in the fact that fungi are eukaryotic, relatively large, and at times multicellular that renders the identification and/or isolation of any specific immunogenic components with protective potential. This in addition to the difficulty to define the candidate population to be vaccinated or the specific fungal pathogen for a vaccine in a given compromised population. Nevertheless, attempts to immunize against fungal agents that cause superficial or systemic mycoses have been made [see reviews and chapters by Casadevall (2001), Cutler et al. (2001), Deepe (1997; 2001), Dixon et al. (1998), and Kirkland and Cole (2001)]. The vaccination studies were based on observations from extensive and in-depth investigations on the immune responses elicited by fungi and the role of the different components of the immune system, as summarized in several reviews by Casadevall et al. (1998) and Stevens et al. (1998), mostly involved experimental animal models with few reaching the stage of clinical human trials.

2 EXPERIMENTAL ANIMAL STUDIES

2.1 Immunizations Against Systemic Mycoses

2.1.1 Deep-Seated Candidiasis

The genus *Candida* includes about 200 species of which about 20 (de Hoog et al. 2000) are known to cause diseases in humans and animals. The major pathogenic species, besides *Candida albicans*, include *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. krusei*, *C. pseudotropicalis*, and the recently emerging new pathogens, such as *C. glabrata*, *C. lusitaniae*, or *C. dubliniensis* (Edwards 2000; Segal and Elad 1998). Extensive studies during the last decade, as summarized by Romani (2002) provide detailed in-depth information on defense systems against candidiasis. It is currently believed that the innate immunity to *Candida* involves several types of cells—PMN, monocytes and macrophages, lymphocytes, and also endothelial and other cells, as well as humoral elements such as complement, cytokines or chemokines, and opsonins (Romani and Kaufman 1998). The role of the different T cells has also been more defined, with differentiation between the Th1, eliciting the protective, and Th2, the nonprotective responses (Romani 1999). The major role that can be assigned to cytokines has also been highlighted (Mencaci et al. 1995). This may open the way for combination therapy of cytokines and specific antifungal drugs.

Attempts to induce protective immunity to candidiasis by active or passive vaccination with the etiological agent or its

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products, and by transfer of serum or cells from immunized donors to nonimmunized recipients have been reported in the literature for several decades, and have been summarized previously (Segal 1987; 1991) describing various immunization protocols and a variety of immunogens including live and killed fungi and subcellular components. Conclusions of the summarized studies indicated that immunizations with live organisms afford partial protection but bear the risks inherent to live vaccines, and that killed fungi or various subcellular fractions may induce a partial protection as well.

Segal and colleagues performed extensive studies on the ribosomal fraction of *C. albicans* as immunogen [reviewed in Segal (1987; 1991)]. Their studies revealed that the *C. albicans* ribosomes were protective against experimental challenge with *C. albicans* and some other *Candida* spp., in naïve and compromised animals, and elicited specific cell mediated and humoral immune responses. Based on this background, Normier et al. (1987) found that by supplementing *C. albicans* ribosomes with a cell wall fraction from *Klebsiella pneumoniae*, an adjuvant permissible for human use augmented the efficacy of the vaccine. A later study performed by the Segal group (Ekstein et al. 1997) approached the issue of adjuvant that could be used in humans. In the studies described previously incomplete Freund's adjuvant (IFA) was used, an adjuvant not suitable for human use, they explored the possibility of using liposomal particles as carriers and possible adjuvants. The efficacy of ribosomes incorporated into liposomes, prepared by two different methods, with and without addition of lipid A was compared to that of ribosomes + IFA in protecting mice against a challenge with a lethal dose of *C. albicans*. The results indicated that the liposomal preparations were as effective as the previously used immunogen − ribosome + IFA. This study showed that *C. albicans* ribosomal particles could be used in humans.

Mathews et al. (1988) pointed to the importance of an antibody to a specific candidal antigen found during disease, which the authors believed could be useful in treating and preventing systemic candidiasis. The observations of Mathews and coworkers in the 1980s, indicating a possible role for anti-*Candida* antibodies in resistance to systemic candidiasis, have been followed by a series of studies in which a specific *Candida* antigen, the heat shock protein 90 (HSP90) was identified as an immunodominant antigen in systemic candidiasis with capability of eliciting protective antibodies (Mathews et al. 1995). Further studies with this antigen led to identification of a specific epitope and production of monoclonal antibodies. Use of molecular engineering enabled production of a human antibody, which is evaluated in human clinical trials (Mathews et al. 2000; Rigg et al. 2001). Moreover, the investigators demonstrated that this antibody might also have a therapeutic value as an adjunct to antifungal therapy (Mathews et al. 2001). Thus, it seems that the anti-HSP90 antibodies are the first anti-*Candida* vaccine or more specifically, immunotherapeutic measure being on the verge of commercialization.

Another promising line of research that focused on antimannan antibodies emerged during the last decade and was led by Cutler et al. (2002). The studies of this group originated from investigations on adherence of *Candida*, where by using an *ex vivo* model they noted that the fungal mannans were involved in attachment to macrophages (Cutler et al. 1990). This was followed by experiments in which mice were immunized with yeast mannan–protein complex encapsulated into liposomes. The mice produced antibodies to mannan and were resistant to a challenge with *C. albicans* (Han and Cutler 1995). More recent studies of this group concentrated on development of specific monoclonal antibodies, more precise identification of specific epitopes, and other refinements of this vaccine (Han et al. 1998; 2000).

An interesting line of research with implications for immunotherapy arose from the studies by Polonelli and coworkers on antibodies to "killer factor." Such antibodies exerted a candidacidal effect and their efficacy was explored in candidal vaginitis (Polonelli et al. 1997) (see Section 2.2.1), as will be described in more detail in the section on mucocutaneous candidiasis.

In the context of feasibility of specific *Candida* components as immunogens, the work of the group in Valencia, Spain, merits to be noted (Gil et al. 1999; Gozalbo et al. 1998). This group focused on the glycolytic enzyme—glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) of *C. albicans*. The GAPDH was found to be a surface antigen that binds to fibronectin and laminin (Gozalbo et al. 1998) and is expressed *in vitro* and in infected tissues (Gil et al. 1999). On the basis of these observations another group, including the author's laboratory has recently undertaken attempts to generate human antibodies to *Candida* GAPDH using a Trimera system (Priel et al. 2002). To summarize, the current state of immunization studies seems to bear the promise that a vaccine is indeed a feasible option.

2.1.2 Cryptococcosis

Cryptococcosis is a generalized mycosis with special predilection for the central nervous system (CNS), including involvement of lungs, bones, and skin, which is caused by the encapsulated yeast *Cryptococcus neoformans* (Diamond 2000). The significance of the AIDS epidemic, which started in the 1980s, and the frequency of cryptococcal infection and its major role in the morbidity of these patients, brought about the continuing increased interest in this fungal infection, particularly in the search for ways of managing or preventing it. Thus, since the 1990s an upsurge of research efforts targeted to explore the immune mechanisms involved in cryptoccosis and attempts to develop a vaccine has emerged, as can be judged by the significant number of research studies published and summarized in recent review articles by Casadevall (2001) and Pirofski (2001). The major host defense mechanisms to protect against cryptococcosis have been believed to be associated with normal function of the cellular system (Murphy et al. 1998). However, antibodies seem to play a role in the defense against cryptococcosis, as

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well. Dromer et al. (1987) reported that a monoclonal antibody to the capsular polysaccharide—glucoronoxylomannan (GXM) administered to mice protected these animals against a challenge with the fungus. Similar observations were made in later studies by other investigators (Mukherjee et al. 1993) showing that monoclonal antibodies to GXM can protect mice from intracerebral infection of *C. neoformans*, the most significant clinical entity of this infection. The promising results of the studies on passive immunization with anti-Cryptococcal antibodies can be seen as a basis for investigations on development of vaccines that would elicit protective antibodies.

As polysaccharides are not considered to be effective immunogens, efforts were made to combine the polysaccharides with a carrier to increase immunogenicity, leading to investigations on conjugate vaccines. Devi et al. (1991) described a *C. neoformans* conjugate vaccine consisting of GXM, and tetanus toxoid (TT) as the protein moiety. This vaccine was protective in mice (Devi 1996) and elicited antibodies, similar to those produced in clinical infection (Casadevall et al. 1992), with ability for passive protection. The GXM-TT vaccine was also explored in humans (Pirofski et al. 1995), revealing that the antibodies elicited have the ability to opsonize *C. neoformans* (Zhong and Pirofski 1996). It should, however, be pointed out that as indicated in a recent review by Pirofski (2001) the GXM-TT vaccine may induce a heterogenic response—both protective and nonprotective antibodies may be elicited (Mukherjee et al. 1995).

In addition, antibodies may be associated with cytokine production or with changes in specific cytokine type production. It was shown that production of the proinflammatory cytokines IL-1beta, IL-2, and TNF-alpha were produced when antibody to GXM was added to human mononuclear cells and *C. neoformans* (Vechiarelli et al. 1998). Another approach to vaccine development, as recently suggested by Pirofski (2001) could be the use of peptide based vaccines, which would mimic the specific GXM epitope that produces protective antibodies. This approach has, however, not yet reached practical application. In summary, the studies of the last decade have brought the feasibility of an anti-Cryptococcus vaccine much closer.

2.1.3 Opportunistic Aspergillosis

The major opportunistic mold infections include aspergillosis and zygomycosis that are caused by the ubiquitous *Aspergillus* and *Zygomycetes* (Denning 2000; Sugar 2000). In view of the lower prevalence of zygomycoses in comparison to aspergillosis, the research efforts targeted at immunization against the former, were accordingly more limited. Thus, the following section will be devoted only to the description of vaccination attempts against aspergillosis. Aspergillosis is a disease of a manifold of clinical entities, including pulmonary, nasal, and paranasal involvement, and a generalized disseminated disease. Aspergillosis gained in importance in modern medicine due to the increase in the number of susceptible individuals, such as leukemia patients (Denning 2000).

In spite of the importance of these infections as causes of human morbidity and mortality, they attracted limited research attention in the area of immunization until the 1990s [summarized by Segal (1991)]. Among the few studies, that of Smith (1972) showed that intravenous inoculation of mice with live spores of *A. fumigatus* induced a dose-dependent protection against challenge with the fungus and no protection was provided by heat-inactivated spores.

The significant advances in the understanding of the functions and mechanisms of the immune system in general, contributed during the last decade to research focused on elucidating the immune response to fungal infections, including aspergillosis. The innate host reaction, when exposed to *Aspergillus* spp., is based on macrophages phagocytizing and killing restive spores, while polymorphonuclear leucocytes and monocytes destroy activated spores and hyphae. Pulmonary macrophages are more efficient in killing the spores than peritoneal ones. Resistance induced in mice was transferable with splenic macrophages but neither with T cells nor with serum (deRepentigny et al. 1993).

Attempts to use *Aspergillus* subcellular components as immunogens were undertaken by Cenci et al. (2000), revealing that crude culture filtrate antigens of *A. fumigatus* protected mice against nasal exposure to the fungus by activating cell mediated components of the immune system. Conversely, the recombinant antigen Asp f2 induced a humoral response and provided no protection. Frosco et al. (1992) inhibited in vitro the activity of elastase, a putative virulence factor of *Aspergillus* spp. by monoclonal and polyclonal antibodies. However, in a subsequent study *in vivo* protection of immunocompromised mice with such antibodies was not achieved (Frosco et al. 1994). While infections caused by *Aspergillus* spp. in mammals are sporadic, in poultry, outbreaks are common (Brown and Jordan 2002), causing heavy economic damages. Richard et al. (1991) examined various vaccination protocols for turkey poults with *A. fumigatus* showing that lesions and mortality can thus be reduced. However, no vaccine has so far been commercialized (Brown and Jordan 2002).

2.1.4 Systemic Mycoses Caused by Dimorphic Fungi

a. Coccidioidomycosis. Coccidioidomycosis is an infection caused by the dimorphic fungus *Coccidioides immitis*, which is endemic in the southwestern United States, northern Mexico, and some parts of Central and South America (Galgiani 2000). Inhalation of *C. immitis* arthrospores results in a high infectivity rate with about 60% of infected individuals being asymptomatic. In most cases, infection leaves the individual resistant to reinfection and is accompanied by development of specific immunity (Galgiani 2000). The major immune mechanism involved is CMI (Magee and Cox 2001) and individuals developing disseminated coccidioidomycosis may have impairment of

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the immune system. The fact that infection leads to development of immunity to reinfection opened the venue of research for induction of acquired immunity by vaccination as a measure for prevention of infection. The high infectivity rate of the organism is an additional incentive for exploration of the possibility of vaccination. Moreover, *C. immitis* is the only fungus to be included in the "Select Agent List" as a potential agent of bioterrorism (Dixon 2001), a fact that further emphasizes the importance of preventive measures. Recently, Barnato et al. (2001) showed the cost-effectiveness of vaccinating children in endemic areas. Research towards vaccine development against *C. immitis* goes back several decades and was reviewed previously by Segal (1987; 1991) and more recently by Pappagianis (2001). Among the earlier studies a central contribution can be attributed to the works of the group of Levine. These investigators used a formalin-killed spherule (FKS) as immunogen in several animal models (Levine et al. 1962; 1965) and their studies led to human trials (Levine and Smith 1967). From the animal studies the investigators reached the conclusion that the spherule vaccine induced protection (Kong et al. 1963). However, the vaccine did not prevent infection (Kong et al. 1964), it merely prevented the dissemination from the lungs. In humans, unfortunately, the dose of the FKS tolerated was not sufficient to induce protectivity (Pappagianis 1993). Therefore, the more recent research with FKS was aimed at overcoming these limitations by fractionations of the immunogen (Zimmerman et al. 1998). Zhu et al. (1997) reported that an alkaline and water soluble extract from *C. immitis* mycelia and spherules, composed of a polymeric (AG2) and a polysaccharidic part induced a T cell response and protectivity. Additional experiments with AG2 included cDNA and recombinant vaccines, and cDNA vaccine with interleukin 12 (IL12) (Jiang et al. 1999). The cDNA vaccine induced protectivity, which was inferior to that of FKS, but was increased by IL12. Kirkland et al. (1998) described two antigens: a proline rich antigen (PRA, similar to AG2) and a 48-kDa T-cell-reactive cytoplasmic protein. Abuodeh et al. (1999) were able to induce protectivity by vaccinating with the recombinant PRA or its cDNA. Li et al. (2001) reported that HSP60, urease and urease DNA stimulated a CMI reaction and showed protectivity.

b. Histoplasmosis. Histoplasmosis, caused by *Histoplasma capsulatum*, is the major nonopportunistic infection of the eastern, central, and midwestern United States, where it is endemic. However, it has also been reported from various regions throughout the world (Deepe 2000). The infection rate in the endemic areas is very high (up to 80% react to histoplasmin) with the great majority of infected individuals being asymptomatic. A minority of the patients, primarily with CMI defects, including abnormal T-helper/T-suppressor cell ratios, run the risk of developing disseminated histoplasmosis. Calcified granulomas, formed during the initial stage of infection may contain viable fungi and reactivate upon debilitation (Deepe 2000). Histoplasma infection generally leaves the patient resistant to reinfection and elicits DTH that is believed to be associated with the induced immunity. The principal immune mechanism responsible for the protectivity is considered to be CMI dependent, with T-cell and cytokine involvement, while humoral response plays a minor role (Allendorfer et al. 1999).

Previous reviews by Segal (1987; 1991) summarized early attempts to induce protection against histoplasmosis describing a variety of immunogens, vaccination protocols, and animal models. Particular attention in the field of anti-*Histoplasma* vaccination should be drawn to the group of Tewari, who employed an *H. capsulatum* ribosomal fraction as immunogen (Feit and Tewari 1974). Their experiments revealed that up to 90% of the vaccinated mice could be protected and that the r-proteins are the primary protection-inducing elements. Although humoral and CMI responses were elicited, the former did not contribute significantly to protectivity (Sharma et al. 1980). Tewari (1985) indicated that the immunogen dose can be reduced and efficacy of vaccination still retained, if the ribosomes are supplemented by other subcellular fractions such as cell wall material.

The 1990s brought renewed interest in the topic of possibilities for anti-*Histoplasma* vaccination (Casadevall 2002; Deepe 2001). The most prominent studies can be attributed to the Deepe group who turned their research efforts towards assessment of various fungal components as immunogens such as cell wall, cell membrane, and a 62 kDa protein from a cell extract (HIS-62). This protein was immunogenic and induced protection in mice (Gomez et al. 1991) and was later identified as a heat shock protein belonging to the HSP60 family (Gomez et al. 1995). The HSP60 was protective in experimental pulmonary histoplamosis (Deepe et al. 1996) and recombinat HSP60 was also protective (Gomez et al. 1995). The research on the HSP60 continues and recent publications (Deepe and Gibons 2001) focus on the identification of the specific T-cell population of importance in the immunity induced by it. The immunogenicity of other HSPs, such as HSP70 and HSP80 has been explored as well (Allendorfer et al. 1996). *H. capsulatum* variant *farciminosum* is the causative agent of epizootic lymphangitis in horses and mules. Two vaccines, one heat-attenuated and another formalinized have given good protective results (Al-Ani 1999).

c. Blastomycosis and Paracoccidioidomycosis. Studies focusing on immunization against *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis*, the aetiological agents of blastomycosis and paracoccidioidomycosis, respectively, are less numerous than those dealing with the previous two fungi and were carried out primarily in the last decade. The most extensive work on *B. dermatitidis* was focused on tests with the WI-1 antigen, a 120 kDa surface protein, targeted by the humoral and cellular branches of the immune system (Wütrich and Klein 2000; Wütrich et al. 2000). A dose dependent protection was induced in mice vaccinated with WI-1, and IL-12 enhanced the stimulatory activity on the CMI system (Wütrich et al. 2000). The main antigen used in immunization experiments against *P. brasiliensis* was a

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43 kDa glycoprotein (gp43) (Pinto et al. 2000). This antigen acts as a virulence factor, probably in the adherence process (Vicentini et al. 1994). It is secreted by the growing fungus (in its yeast form), reacts with sera from infected subjects (Taborda and Camargo 1993) and induced a cellular type immune reaction (Saraiva et al. 1996). In murine experiments, gp43 induced a Th1 CD4 + reaction (Travassos et al. 1995), resulting in interferon γ and interleukin 2 production. A fraction of 15 amino acids (P10) from gp43 was responsible for these reactions and was protective in mice (Taborda et al. 1998). More recently, the gp43 gene was reported to be able to induce a reaction similar to the corresponding antigen and protected mice against intratracheal infection (Pinto et al. 2000). In conclusion, the advanced state-of-the-art of the studies on immune mechanisms involved in histoplasmosis and coccidioidomycosis, and the investigations on detection or identification of fungal components exhibiting protective potential, justify the hope for the development of a vaccine to manage these mycoses. Thus, the prospects for protective immunization seem feasible for these mycoses, albeit, no practical vaccine is currently forthcoming.

2.1.5 *Pneumocystis carinii*

Pneumocystis carinii is a unicellular microorganism (Waltzer 2000), first described in 1909 by Chagas. The taxonomy of this microorganism has been the subject of much debate and it was classified as a protozoan parasite until 1991 but is currently considered as a fungus (Pixley et al. 1991). Exposure to *P. carinii*, as judged by the presence of antibodies is common (Peglow et al. 1990). The microorganism may be found in the lungs of humans and a large variety of animals with intact immune system (Waltzer 2000). *P. carinii* causes pneumonia in immunosuppressed or otherwise debilitated subjects and is considered a major pathogen of AIDS patients. Both the CMI and humoral components of the immune system are activated by exposure to *P. carinii* (Gigliotti and Hughes 1988; Graves et al. 1991) and each, possibly independently, is involved in protection (Roths and Sidman 1993). Several *P. carinii* antigens have been used in immunization experiments. The most frequently used was the major surface glycoprotein (MSG) of 120 kDa, also known as gp120 (Fisher et al. 1991) or glycoprotein A (Gigliotti et al. 1998) which was able to stimulate CMI and humoral systems (Fisher et al. 1991). The ability of this antigen to induce protectivity against *P. carinii* infection has thus far not been established and some authors found it not protective (Gigliotti et al. 1998) whereas others (Theus et al. 1998) showed the opposite. Another antigen group eliciting cellular and a humoral immune reaction, and providing partial protection, consists of 45–55 kDa or 35–45 kDa proteins in rat or human derived strains, respectively (Smulian et al. 2000). In addition, a soluble *P. carinii* antigen (Pascale et al. 1999) and various monoclonal anti-*P. carinii* antibodies (Gigliotti et al. 2002) were found to be protective. Zheng et al. (2001) reasoned that, since $CD4^+$ cell deficiencies in AIDS patients impair successful vaccination against *P. carinii*, an approach based on $CD4^+$ independent immunization should be attempted. These investigators induced significant protection in $CD4^+$ deficient mice by immunizing them with marrow-derived dendritic cells expressing the murine CD40 ligand, pulsed with *P. carinii* antigen.

2.2 Immunizations Against Superficial Mycoses

2.2.1 Superficial Candidiasis

The superficial forms constitute the majority of candidiasis cases involving skin, nails, and mucous membranes (Segal and Elad 1998), and although not life-threatening may pose a problem due to recurrences and therapeutic difficulties in some of the clinical entities. Thus, prophylaxis by vaccination could contribute to the management of these infections. Recent literature on the defense system in cutaneous candidiasis (Sohnle et al. 2002) supports the dogma of the importance of CMI, although highlights additional information on other components such as keratinocytes or Langerhan's cells. As recently summarized by Fidel and Sobel (2002) the defense system in vaginal candidiasis seems to be even more complex, and although it attracted a fair amount of research activity, the protective mechanisms are yet not entirely elucidated. The accumulated information suggests that both local and systemic immunity may play a role with involvement of CMI (particularly T cells), cytokines, and antibodies. The latter observations are the basis for attempts to induce protection by vaccination eliciting anti-*Candida* antibodies against superficial candidiasis (Elahi et al. 2001). It was shown by Han et al. (1998) that monoclonal antibodies to *C. albicans* mannan epitopes have an effect on vaginal candidiasis in mice. A protective role for antimannan antibodies in experimental *Candida* vaginitis in rats was also shown by De Bernardis et al. (1997). These investigators indicated that antiaspartyl proteinase antibodies might have a protective role as well (De Bernardis et al. 1997). The efficacy of antibodies to yeast-killer toxin (Polonelli et al. 1996) was also evaluated in an experimental vaginal model in rats.

2.2.2 Dermatophytoses

Dermatophytes, the etiological agents of dermatophytoses, comprise 40 species assigned to three genera, and are ubiquitous keratinophilic molds, which thrive in soil using keratinous debris or parasitize animal and human keratinous tissues (Hay 2000). Dermatophytoses are among the most frequent fungal infections. Currently available antifungal therapy, although effective in treating most cases of dermatophytoses, does not prevent recurrences and, in some clinical forms, must be continued for prolonged periods. Animal dermatophytoses are also frequent infections, and may involve wild, farm, or domestic animals, including pets, and can be a source of zoonotic infections, this in addition to their economic implications. Thus, their prevention by vaccination could be desirable.

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Most early investigations of the immune response to dermatophytes were based on crude or partially purified antigens. More recently, however, the activity of well-defined antigens has been investigated (Woodfolk et al. 1996). Two proteins extracted from *T. tonsurans*, Trit t 1 and protein IV, produced both delayed and immediate cutaneous hypersensitivity, thus indicating that one antigen may influence both the cell mediated and humoral components of the immune system (Woodfolk and Platts-Mills 1998). Furthermore, Slunt et al. (1996) demonstrated that the nature of the response depends on the host.

While it is generally accepted that the major defense mechanism against dermatophytoses is the cell-mediated system, the function of the humoral system is less clear and it is even believed that the presence of circulating antibodies may antagonize the activity of the cell mediated reaction (Woodfolk and Platts-Mills 1998). However, Pier et al. (1993) maintain that humoral reaction contributes to protection. These discrepancies might result from variations in the dynamics of the immune response in different host–parasite combinations. Thus, in cats that recovered from *Microsporum canis* infection, no differences in antibody levels between culture negative and persistent carriers were found (DeBoer and Moriello 1993). Keratinocytes produce various cytokines, some leading to the activation of T lymphocytes (Cella et al. 1997), while others promote antibody formation (Romani et al. 1993). Consequently, keratinocytes might have a pivotal role in determining the direction of the immune reaction, and thus the clinical characteristics of the infection. Since, the humoral and cellular responses are inversely related, it is of great importance for vaccine efficacy to stimulate primarily the CMI component. It was noted by Smith and Griffin (1995) that antigens obtained from germinating spores and early hyphal growth are better candidates for vaccines inducing a cell mediated immune response than those obtained from mature fungal cultures. The possibility of adding to vaccines cytokines capable of stimulating the cell-mediated immunity has been proposed by Ramshaw et al. (1992).

Two characteristics of dermatophytoses indicate the possibility of successful immunization: (a) recovered animals rarely become infected again, and if they do, lesions heal more quickly than in naïve hosts and (b) immunity of recovered animals extends to dermatophyte species other than the original infective agent (Smith et al. 1992). Nevertheless, such attempts have not been numerous, involving primarily zoophilic dermatophytes. Some of these studies were confined to experimental models in laboratory animals, while others reached the stage of experimentations at the level of the natural host, such as *T. verrucosum* in calves or *M. canis* in foxes (Sarkisov Nikoforov 1988). Several vaccines have been developed to commercial products. Prophylaxis of *T. verrucosum* infections in cattle was a major target. Studies toward this goal were reported as early as the 1950s and were reviewed in detail previously (Segal 1989). Such trials, which resulted in the development of an effective vaccine, used on a large scale were undertaken by Sarkisov et al. (1971). These investigators used a live vaccine, LTF-130, composed of an attenuated *T. verrucosum* strain. Prolonged follow-up (Sarkisov et al. 1983) in field experiments revealed that the immunity induced was long lasting. Large-scale vaccination programs with the LTF-130 based vaccine in various countries led to a significant reduction of the incidence of cattle ringworm (Bredahl and Gyllensvaan 2000; Gordon and Bond 1996; Sarkisov and Nikoforov 1988). The LTF-130 vaccine mounted a CMI response and interferon-γ production (Lund et al. 2001). Further vaccines based on the same concept were developed, including *T. mentagrophytes* for fur animals and *T. equinum* for horses (Sarkisov and Nikoforov 1988). Additional commercial vaccines include, among others, inactivated preparations against *M. canis* (DeBoer and Moriello 1995) and attenuated preparations against *T. mentagrophytes* (Rybnikar et al. 1998).

Several other investigational vaccines were tested on target hosts. An inactivated *T. equinum* (Pier and Zancanella 1993) successfully protected horses against contact exposure. A *T. verrucosum* ribosomal vaccine induced CMI and humoral reactions, and significantly reduced the duration of lesions on naturally infected calves (Elad and Segal 1995). Experiments in laboratory animals included a study of Pier et al. (1995) with a multivalent inactivated *M. canis*, *M. gypseum*, *T. equinum*, *T. mentagrophytes* var. *mentagrophytes* preparation that protected guinea pigs against *M. canis*. Elad and Segal (1989; 1994) vaccinated guinea pigs with an *M. canis* ribosomal preparation and induced a cell mediated as well as humoral reaction in these animals. Upon challenge with the fungus, infection periods were reduced significantly in vaccinated guinea pigs. In addition to its immunizing capacity, the *M. canis* ribosomal fraction was shown to have therapeutic activity in naturally infected puppies (Elad and Segal 1991). In conclusion, it seems that at present only dermatophyte preparations from a few zoophilic species, particularly *T. verrucosum*, may be considered as practical vaccines of veterinary use. These vaccines, in addition to their economic impact, may contribute indirectly to the reduction in the prevalence of human dermatophytoses caused by those dermatophyte species.

3 HUMAN TRIALS

The few, difficult to evaluate, anecdotal reports on experiments to prevent mucosal or cutaneous and nail infections in humans by specific vaccination or by nonspecific boosting of the immune system were reviewed previously (Segal 1991). The most extensive and systematic human trials were conducted with the killed *C. immitis* spherule vaccine. Following the successful results obtained in animal models, including monkeys, first human trials were initiated in the mid-1960s, evaluating initially the safety of the vaccine and the immune response elicited by it (Levine and Smith 1967; Pappagianis et al. 1967). From these initial studies it could be concluded that: (a) no generalized adverse reactions to the vaccine were observed, but at a dose of over 5 mg a local

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transient tissue reaction at the site of injection was noted, (b) vaccination elicited, albeit not always regularly, dermal delayed hypersensitivity (DTH) to coccidioidin; and (c) no anticoccidioidal antibodies were produced. Later studies (Williams et al. 1984; 1985), involving larger group populations and different vaccine doses administered IM were conducted with the goal of investigating in-depth the immune response elicited by the vaccination. It could be concluded that vaccination led indeed to the development of DTH in about 50% of the vaccines, and to the production of anticoccidioidal antibodies in about 16% of vaccinated subjects. In addition, this trial also evaluated the safety of the vaccine, and confirmed the previous findings, that no systemic side effects were noted, but that in some individuals (3%) receiving the higher dose (3.5 mg) there was a local reaction. Further evaluation of the safety of the vaccine was undertaken in an even larger group (1245 patients) (Williams et al. 1985). In this group, too, in about 7.5% of the tested individuals an adverse reaction was noted. In 1980 a large-scale, double blind clinical trial (Galgiani et al. 1986) was initiated to test whether the spherule vaccine reduces morbidity due to coccidioidomycosis. The study continued until the fall of 1984. Five thousand two hundred twenty-eight persons were screened for participation in the study, 2654 were excluded due to positive coccidioidal skin tests or other reasons, and 2834 were included. These were divided into two groups of 1417 each, to receive either the vaccine or placebo (saline). The vaccination protocol included three IM injections of 1.75 mg of vaccine material. The first two injections were given at 1-week intervals, and the third at 6 weeks after the second. The evaluation of that study (Pappagianis 1993) revealed, unfortunately, that no significant differences in the morbidity from coccidioidomycosis between the vaccinated and nonvaccinated groups were noted. The possible explanation for the failure could lie in the nature of the vaccine. As indicated in Section 2, the vaccine did not prevent infection; it merely prevented dissemination from the lungs. Since, the vaccine preparation is composed of whole spherules, which may be responsible for the adverse local reaction observed in a part of the vaccinated human subjects, relatively low vaccine doses had to be used that seem to be not sufficiently protective. Hence, the latest studies focused on identification of specific subcellular antigenic components with protective ability (see Section 2.1.4), however, these investigations have not reached yet the stage of clinical human trials.

The last few years were characterized by additional attempts to investigate vaccination or immunotherapy in humans. Among these are the studies of Matthews et al. (2000) who developed a monoclonal antibody to HSP90 of *C. albicans*. This antibody, which revealed protective ability in experimental models, as detailed above (see Section 2.1.1) has reached the stage of human evaluation. It is being assessed in humans, including evaluation of its role in immunotherapy of candidiasis in combination with antifungal chemotherapy (Matthews et al. 2000). Another vaccine preparation against a systemic fungal infection which was tested in the 1990s in humans is the anti-*C. neoformans* GXM-TT vaccine (Pirofski et al. 1995). This vaccine is based on the *C. neoformans* capsular polysaccharide—GXM conjugated to the TT. It was shown that the antibodies elicited by this vaccine have the ability to opsonize *C. neoformans* (Zhong and Pirofski 1996). It seems, however, that the efficacy of this vaccine preparation is hampered by the inconsistent response it elicits. It is of interest to mention the study of Fleuridor et al. (1998) who found that a human monoclonal anti-GXM antibody prolongs survival of mice infected with *C. neoformans*.

4 CONCLUSIONS

This chapter reviewed recent literature in the field of immunization attempts against superficial and systemic fungal infections in humans and animals. Summarizing the available information indicates the following major points: (a) the last decade has been characterized by an understanding of the importance of vaccination and/or immunotherapy as tools for prevention and/or management of mycoses. Hence, extensive in-depth studies, using state of the art immunologic and molecular biology technology have been conducted, aiming to investigate the immune responses involved in various mycoses. These studies led to significant advances in the elucidation of the complexity of the immune responses, defense mechanisms, and antigenic components and thereby laid the foundation for development of vaccines, (b) the systemic mycoses attracted the majority of the research efforts toward eventual development of vaccines. The most extensive and systematic studies in experimental animal models were carried out against systemic candidiasis, cryptococcosis, histoplasmosis, and coccidioidomycosis, (c) although no clinically applicable vaccines against fungal infections are available, the research efforts in this field, led in some cases, to immunization attempts involving humans. These included, primarily, the *C. neoformans* conjugate vaccine consisting of the capsular polysaccharide glucoroxylomannan (GXM) conjugated with TT, and the recent experiments with antibodies to the HSP90 of *C. albicans* as adjunct immunotherapy in combination with specific antifungal treatment. Previous trials to immunize humans against coccidioidomycosis with formalinized killed spherule vaccine, although not leading to a decrease in morbidity, pointed to the feasibility of prophylaxis, and provided a starting point for the search of more defined immunogenic components, and (d) in the area of superficial mycoses, in addition to the already established antidermatophyte vaccine for veterinary use, experimentations involving immunization against superficial candidiasis, such as vaginal or oral infections have been undertaken. It can be thus concluded, that immunization against mycoses seems to be a feasible endeavor, hopefully, resulting in the development of antifungal vaccines in the foreseeable future.

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Fungal Allergy

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1 INTRODUCTION

Allergy is the exaggerated response of the immune system to foreign proteins such as environmental allergens including mold, pollen, and other allergens (Blumenthal and Rosenberg 1999). The allergic conditions most frequently recognized are allergic rhinitis, conjunctivitis, and bronchial asthma. The immune response in atopy results from the interaction of the host with an allergen and other modulating environmental factors. Not all individuals develop allergy even after extensive exposure to allergens suggesting a role for genetic predisposition in hypersensitivity diseases. Allergen has been defined as the substance that is involved in atopy, an adverse immune reaction resulting from the induction of specific Immunoglobulin E (IgE). Exposure to allergens can be by inhalation, contact, ingestion, and by injection. The response to an allergen is determined by its structure and nature, host factors such as genetical, predisposition and underlying diseases, and environmental factors, including pollution and high levels of allergen concentrations.

Fungi are present in both indoor and outdoor air, although their concentrations in the outdoors vary depending on the climate and other factors. The concentration and the presence of indoor allergens vary substantially and are dependent on moisture content, ventilation, and the presence or absence of carpets, pets, and house plants (Gravesen 1999). Clinically, the presenting symptoms of fungal allergy are not different from other allergies caused by other agents and include sneezing, coughing, wheezing, and shortness of breath. Reversible pulmonary airway obstruction, angiedema, urticaria, and even anaphylaxis may be present in these patients. Inflammation is an important feature of these conditions that include complex histological reactions in different tissues. The inflammatory condition results from antigen-induced immune response involving both cellular as well as humoral components of the immune system.

2 ALLERGIC DISEASES CAUSED BY FUNGI

Fungi are eukaryotic, filamentous, and mostly spore-bearing organisms. Fungi exist as saprophytes or as parasites of animals and plants. Allergy to fungi follows the same biological phenomena as allergy to other environmental allergens. Fungi grow on most substrates even in adverse conditions. Large number of airborne spores is usually present in outdoor air throughout the year and the number may exceed that of the pollens at times (Burge 1989). The major indoor allergens constitute house dust containing mites, epithelial cells, dandruff, cats, dogs, cockroaches, and fungi (Gravesen 1985). Depending on the moisture content of the internal air, the fungal spores also may vary considerably in variety and prevalence (Gravesen 1999).

Fungi are associated with a number of allergic diseases in man. The prevalence of fungal allergy is estimated to be about 30% among atopic individuals and about 6% in the general population. The major allergic manifestations induced by fungi are allergic asthma, rhinitis, allergic bronchopulmonary aspergillosis, and hypersensitivity pneumonitis (Burge 1989; Kurup and Fink 1993). These diseases can result from exposure to a variety of antigens present in the fungal spores and hyphae. A number of fungal spores can reach the alveolar surface through breathing because of their small size. Soluble antigens liberated from fungi also will be inhaled. The spores and antigens mediate diverse allergic reactions depending on the genetical and other predisposition of the subjects.

Some genera of airborne fungal spores such as *Alternaria*, *Aspergillus*, *Penicillium*, and *Cladosporium* are distributed throughout the world. The airborne spores of these fungi are generally considered as an important cause of both allergic rhinitis and allergic asthma (Vijay et al. 1998).

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2.1 Allergic Asthma

Allergic asthma is a chronic inflammatory airway disease caused by the sensitization to allergens belonging to a number of different sources including fungi (Kurup and Banerjee 1997). The disease is characterized by airway mast-cell degranulation, epithelial cell disruption, mucus hypersecretion, subepithelial collagen deposition, and interstitial inflammatory response. The inflammatory cells in the infiltration represent eosinophils and lymphocytes. Asthma is also characterized by airway obstruction resulting from the inhalation of allergens. There are two types of responses detected in asthma, the acute asthmatic response occurs within minutes after the exposure to allergens and is mediated by the release of mediators such as histamine, tryptase, leukotrienes, etc., which induce airway mucosal edema, smooth muscle contraction, and mucus release (Krishna et al. 2001). Antigen binding to the IgE on the surface of the mast-cells leads to cross-linking and activation resulting in the release of mediators, which may contribute to the airway smooth muscle hyperresponsiveness, and eventual anaphylaxis. The late phase reaction occurs in 3–8 h and results from the cytokine networks including IL-4, IL-5, IL-13, and chemokines including RANTES, eotaxin, MCP-1, MCP-2, MCP-3, and MIP-α, released by lymphocytes and ancillary cells. These products and the Th2 cells coordinate a cellular influx of activated eosinophils, neutrophils, macrophages, and T-lymphocytes to the late asthmatic response. This further releases more mediators from these activated inflammatory cells in the cause of late phase response airway obstruction and persistent asthma. The fungi capable of causing allergic asthma are listed in Table 1. To this list additional new antigens are being added with increasing frequency. The most predominant fungi associated with allergic asthma are *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* and will be discussed below.

2.2 Allergic Rhinitis

Allergic rhinitis is a chronic disease defined as a clinical hypersensitivity of the nasal mucosa to allergens mediated through IgE antibodies (Baroody and Naclerio 2001; Kurup and Kumar 1991). Rhinitis occurs in about 15–20% of the population. Low dose of exposure to the offending antigen over a period of many years leads to the development of allergic rhinitis. Asthma and allergy are predisposing conditions for rhinitis. The low dose antigen exposure in the initial stage of the disease results in the production of specific IgE antibodies. There are a number of fungi involved in causing rhinitis and more frequently encountered fungi are *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Curvularia*, and *Penicillium*. The cross-linking of IgE molecules on the surface of mast-cells after allergen binding, leads to mast-cell degranulation and release of mediators contributing to the clinical manifestations of the disease, namely sneezing, pruritis, rhinorrhea, and nasal obstructions.

Table 1 Genera of fungi frequently associated with allergy

Alternaria	*Drechslera*	*Saccharomyces*
Aspergillus	*Epicoccum*	*Scopulariopsis*
Aureobasidium	*Fusarium*	*Stachybotrys*
Botrytis	*Gliocladium*	*Stemphylium*
Candida	*Helminthosporium*	*Trichoderma*
Cephalosporium	*Paecilomyces*	*Trichophyton*
Cladosporium	*Penicillium*	*Trichothecium*
Curvularia	*Phoma*	*Ulocladium*

2.3 Allergic Bronchopulmonary Mycoses

Allergic bronchopulmonary mycoses (ABPM) develops from sensitization with fungi present in the environment. The development of ABPM depends on the mode and frequency of exposure to the antigens. Although several fungi have been implicated in ABPM such as *Candida albicans*, *Curvularia*, *Dreschlera*, *Geotrichum*, *Helminthosporium*, etc. by far the most predominant organism associated with ABPM is *Aspergillus fumigatus* (Kurup and Apter 1998). Sensitization with *A. fumigatus* allergens occurs in combination with other aeroallergens and the disease is more prevalent in asthmatics and in patients with cystic fibrosis. Allergic bronchopulmonary aspergillosis (ABPA) demonstrates specific clinical and immunological characteristics. The ABPA shows peripheral blood eosinophilia, immediate cutaneous hyperreactivity to *Aspergillus* antigen, elevated total serum IgE, precipitating antibody to *Aspergillus* antigen, specific IgE and IgG antibodies to the antigen and increased serum IL-2 receptor concentrations (Kurup et al. 2000). *A. fumigatus* grows saprophytically in the bronchial lumen resulting in persistent bronchial inflammation leading to bronchiectasis in patients with asthma. The bronchiectasis is frequently proximal in the central two-thirds of the lung field on high-resolution tomographic examination. If not diagnosed and treated early, ABPA becomes a chronic disease with marked pulmonary disability.

2.4 Allergic Sinusitis

Sinus disease due to fungi cause several clinical and pathological syndromes, which include fungal ball production and allergic sinusitis (Kurup and Banerjee 1997). The allergic fungal sinusitis is usually chronic and affects multiple sinuses without tissue invasion. Several fungi have been implicated in allergic fungal sinusitis the most important ones are *Aspergillus*, *Curvularia*, *Alternaria*, and *Bipolaris* (Brummund et al. 1986) Majority of the patients demonstrate nasal polyposis, asthma, and rhinitis. Mucoid impaction resulting in plugs containing fragments of the fungi similar to those seen in the sputum plugs of patients with ABPA. The

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sinus exudate may contain eosinophils, Charcot–Leyden crystals, epithelial cells, and fungal elements. Radiologically progressive thickening and clouding of several or all paranasal sinuses occurs in most patients. Patients show cutaneous hypersensitivity to specific allergens and marked elevation of serum total IgE and specific IgE and IgG antibodies.

2.5 Hypersensitivity Pneumonitis (HP)

Hypersensitivity pneumonitis, an allergic lung disease, is caused by the inhalation of a number of antigens including fungal antigens (Kurup and Fink 2002). The common fungi associated with hypersensitivity pneumonitis belong to the genera *Aspergillus* and *Penicillium*. The species recognized as causing HP are *A. terreus*, *A. clavatus*, and *A. fumigatus*, while *Penicillium frequentans* and *P. casei* are the common organisms associated with HP. These organisms grow in the ventilation systems of buildings and the inhalation of allergens leads to the development of allergy. The acute forms have no special characteristics and may be confused with respiratory viral infections. On repeated exposure, the disease becomes chronic and the patient may develop symptoms 4–8 h after exposure to antigens. Recurrence occurs each time when exposed to the antigens. Continuous exposure to the antigens results in intense inflammatory reaction in the lungs leading to irreversible lung damage in chronic and untreated patients. Lung biopsies often show interstitial fibrosis with granuloma formation and alveolar wall thickening due to infiltration of lymphocytes, plasma cells, and eosinophils. The most characteristic features of HP is the presence of serum precipitating antibodies and antigen induced lymphocyte stimulation. Frequently, no IgE antibody response was noted in these patients.

3 MECHANISMS OF FUNGAL ALLERGY

The immune response in type 1 allergy is mediated by both T and B cells (Blumenthal and Rosenberg 1999; Krishna et al. 2001). IgE forms a critical link in the immediate hypersensitivity reactions in humans. Total and antigen specific IgE is enhanced in allergic asthma, allergic rhinitis, allergic sinusitis, and in allergic bronchopulmonary mycoses. IgE binds to Fc epsilon receptor 1 (FcεRI) and mediate anaphylaxis. The components of the human allergic response including IgE are highly oriented toward mucosal surfaces particularly that of the respiratory tract. Compared to serum concentration of IgE, markedly high level was detected in the respiratory and nasal secretions (Krishna et al. 2001). This is further confirmed by the presence of IgE bearing plasma cells in the histological sections of the respiratory mucosa.

The T-cells upon activation through allergen presentation lead to a Th2 pathway with secretion of several very specific cytokines, chemokines, nuclear factors, etc. and contribute towards the overall presentation of the type 1 allergy in these diseases (Kurup et al. 1998). The IgE synthesis involves a number of signaling following both cognizant and non-cognizant interaction between T and B-lymphocytes. The specific IgE formed against the allergens bind to the receptors on the basophils and mast cells. On further interaction of the allergens with these IgE lead to the release of vasoactive components from these cells, which in turn results in localized or systemic anaphylaxis.

3.1 Role of B-Cell in Allergic Responses

IgE antibody in humans participates in immediate hypersensitivity through its ability to bind to specific ligands, which make it possible for IgE to trigger both afferent and efferent arms of immune reactivity. In comparison with other immunoglobulins, IgE needs only very low doses of the allergens. Three receptors that can bind to IgE and trigger anaphylactic reaction have been identified. These receptors are FcεRI, FcεRII, and Galectin 3 (Saxon et al. 2001).

3.2 IgE in Antigen Capture and Presentation

IgE antibody plays a major role in the antigen capture and presentation to lymphocytes. FcεRI and II are expressed on various antigen-presenting cells. Antigen bound to the IgE on the cells can be presented directly to B-cells or processed and presented to antigen specific T-cells. The FcεRII (CD23) mediated focusing of antigen and subsequent processing and presentation is not only IgE dependent, but also IgE restricted. FcεRII bearing cells treated with antigen specific IgE antibody were 1000-fold more effective than untreated B-cells in focusing and presenting low concentrations of antigens to T-cells (Van der Heijden et al. 1993). IgE facilitated antigen presentation by activated B cells to antigen specific $CD4^+$ T-cells biases towards a Th2-type response producing IL-4, IL-5, and IL-13 cytokines.

The other major antigen presenting cells express both FcεRI and FcεRII are follicular dendritic cells (FDC). The FcεRII on the FDC in the secondary follicle germinal center can provide antigen presentation to B-cells where they undergo antigen stimulation, clonal expansion, and affinity maturation of their immunoglobulin receptors. The FDC can provide antigen presentation through FcεRI and II and interact through cell–cell contact with T-cells and produce cytokines, which may further enhance the immune response. The FDC also can utilize their FcεRII for capturing antigen antibody complexes containing IgE and can be processed and presented to the special T-cells present in the germinal center. The IgE mediated capture of antigen by FDC provides a prolonged immune response. Similarly the specialized dendritic cells present in skin nasal mucosa and lungs called Langheram cells (LC) also play a major role in the IgE mediated immune response. Langheram cells are capable of functioning as antigen presenting cells, but are also capable to bind to IgE through FcεRI and RII and Galectin 3. The FcεRII (CD23) receptors are rapidly cleaved from the cells and the

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soluble CD23 participate in the lymphocyte stimulation and IgE regulation. Thus, LC like FDC plays a major role in the allergic responses.

3.3 T-cells and Cytokines in Asthma and Allergy

The information gained during the last two decades indicates that T-cells play a major role in the inflammatory process in allergy and asthma. A marked increase in the number of T-cells has been demonstrated both in the bronchoalveolar lavage and in the lung biopsies. Two distinct types of T-helper cells Th1 and Th2 have been characterized based on their cytokine profile (Mosmann and Coffman 1989). Both subtypes secrete IL-3 and GM-CSF, while Th1 subtypes preferentially produced IL-2, interferon-gamma (IFN-γ), and TNF-β, while Th2 subtypes showed IL-4, IL-5, IL-9, and IL-13. IL-2 induces T-cell activation, while IFN-γ inhibits B-lymphocyte activation and IgE production. These cytokines are important in the delayed hypersensitivity reactions in chronic granulomatous disease such as tuberculosis, leprosy, and sarcoidosis. The Th2 cytokines IL-4 and IL-13 are important in IgE isotype switching by B-cells. IL-13 is also associated with enhanced mucus secretion by airway epithelial cells (Ford et al. 2001). IL-5, IL-3 and GM-CSF are important in the eosinophil recruitment, maturation, and survival.

Bronchoscopic studies revealed preferential upregulation of Th2 cells in allergy and asthma. The asthmatic airway showed a marked increase in cells showing mRNA of IL-3, IL-4, IL-5, IL-13, and GM-CSF, but no IFN-γ. Following allergen challenge there was a further increase in the cells exhibiting IL-4, IL-5, and IL-13 (Robinson et al. 1993). The allergen challenge also resulted in a remarkable decrease in the IFN-γ and IL-2 secreting cells in the bronchoalveolar lavage. The evidence indicates that T-cell responses are skewed to a Th2 typed response in asthma. There are a number of costimulatory molecules actively participating in the T-cell activation. The processed antigen by APC will be presented to $CD4^+$ T-cells via MHC class II molecules. The interaction of T-cell receptor and MHC class II molecules is followed immediately by the costimulatory molecules such as CD80 and CD86 on the B-cells and APCs interacting with CD28 on the T-cells. The activation of CD28 results in the transcription of Th2 cytokines. However, a molecule closely related to CD28 located on T-cell surface, CTLA-4 on activation inhibits Th2 cytokine switch. A definite Th2 shift is detected in allergen stimulated T-cells *ex vivo* where CD80 and 86 interaction with CD28 occurs, which can be inhibited by CTLA-4 (Saxon et al. 2001).

3.4 Chemokines in Asthma and Allergy

Chemokines are small molecular weight cytokines, control and direct the migration and activation of various leukocyte populations (Rothenberg 2000). They fall into two sub-families based on whether the two amino terminal cysteine residues are immediately adjacent (C—C) or separated by one amino acid (C—X—C). The former is called α-chemokine, while the latter are known as β-chemokines. The α-chemokine are produced predominantly by T-cells and represent monocyte-chemotactic protein (MCP) 1, 2, and 3, RANTES, monocyte inflammatory proteins (MIP) 1α and 1β and Eotaxins. These molecules act predominantly on monocytes, eosinophils, basophils, and neutrophils and are directly associated with allergic response. The chemokine receptors such as CCR1, CCR2, CCR3, and CCR8 are involved in Th2 mediated immune responses and hence, have major role in allergy and asthma (Lucas 2001). As these chemokines control and direct the migration and activation of various leukocyte populations by appropriate targeting of chemokines may lead to new ways of controlling the inflammation in asthma.

3.5 Role of Eosinophils in Allergy and Asthma

Because of its presence in the peripheral blood and sputum of asthmatic patients, eosinophils have been recognized long before T-cells and mast cells were recognized as a major player in the pathogenesis of asthma. The production of eosinophils in the bone marrow is regulated by a number of growth factors such as GM-CSF, IL-3, and IL-5. Several other factors such as RANTES, Eotaxin, and MIPs have been identified as major chemokines associated with recruitment, survival, and activation of eosinophils (Weller 2001).

Eosinophils contain a number of granules, which store preformed mediators. These granules include eosinophil chemotactic factor (ECP), major basic protein, (MBP), eosinophil peroxidase (EPO), and eosinophil derived neurotoxin (EPN). Eosinophils are sources for a number of factors including cytokines and chemokines associated with asthma pathogenesis. These include IL-3, IL-4, IL-5, IL-6, GM-CSF, IL-8, TNF-α, macrophage inflammatory peptide-1α (MIP1-α), TGF-α, and Beta. Other mediators include postaglandins, platelet activating factors, and a number of enzymes. The bronchial hyperreactivity in asthmatic patients can also result from eosinophils and its products. A marker of eosinophil activation, ECP, reported from airway and serum has been correlated with disease activity particularly acute exacerbation (De Monche et al. 1985). Eosinophil and airway epithelial cell interaction lead to epithelial damage in asthmatics.

3.6 Mast Cells

Mast cells are important effector cells in allergy and asthma. Mast cells are ordinarily distributed throughout normal connective tissues, where they often lie adjacent to blood and lymphatic vessels and beneath epithelial surface exposed to environment such as lung, gut, and skin. The mast cells contain granules, which secrete tryptase, chymase,

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carboxypeptidase, and Cathepsin-G like enzymes. The mast cells are under the influence of T-cell cytokines, particularly IL-6 and IL-9 or fibroblast derived growth factors.

Basophils and mast cells on appropriate stimulation elaborate a number of biologically active mediators. Some of these mediators are preformed in cytoplasmic granules such as histamine, heparin, and proteases, while others are released upon cellular activation. They also produce lipid mediators such as prostaglandins and leukotrienes on appropriate stimulation of the cells. Mast cells can transcribe and secrete cytokines such as IL-1, 2, 3, 4, 5, 6, 8, 10, 13, 16, GM-CSF, TNF-α, and a number of C—C chemokines. These products are released when the cells are activated. These secretory products are a clear indication on the role of mast cells in the pathophysiology of allergic and immunological diseases and or host defense (Galli 2000). The evidence indicates that the release of cytokines by mast cells contribute to the development of chronic mucosal inflammation through the recruitment of T-cells and eosinophils. Through an IgE mediated mechanism, mast cells release preformed and newly generated mediators that contribute to the chronic mucosal inflammation in asthma.

3.7 Other Factors and Their Role in Allergy and Asthma

Nuclear factor-kβ (NF-kβ) is considered to play a major role in the regulation of cells activity. NF-kβ is an important transcription factor that regulates several inducible genes including inducible nitric oxide (iNOS), various adhesion molecules, cytokines, and growth factors. This factor present in the cytoplasm of cells is an inactive form on activation undergo phosphorylation and localizes in the nucleus, where it binds to specific kβ recognition elements on the promoter region of the target genes. In atopic asthmatics an increased immunoreactivity for NF-kβ has been demonstrated in endothelium and submucosal mast cells (Saxon et al. 2001).

A number of adhesion molecules associated with recruitment of inflammatory cells have been identified. Following the stimulus to the endothelium, leukocytes adhere to the endothelium. These cells eventually cause diapedisis and migrate from the vessels to perivascular space. These adhesion molecules include ICAM-I, ICAM-2, VCAM-1, etc., are further up regulated on mast cells or endothelial cells by selected cytokines.

4 FUNGAL ALLERGENS

Relevant fungal allergens are important in the diagnosis of allergy due to fungi by demonstrating IgE antibody in the sera of patients or by immediate wheal and flare skin test reactivity. The diagnosis of allergic disease is mainly based on clinical symptoms of the patients, skin test reactions, detection of allergen specific serum IgE antibodies (RAST, ELISA), and in some cases inhalation challenge using fungal antigens (Portnoy et al. 1987). However, the effective *in vivo* and *in vitro* diagnosis of fungal allergies is dependent on the availability of well-characterized allergen preparations. Fungi are highly variable both in their morphology and in their antigenic makeup. The wide-spread antigenic cross-reactivity among related fungi is a major concern. With some fungi it is almost impossible to grow two consecutive cultures with similar antigenic profiles (Aukrust 1979; Kurup et al. 1977). Factors contributing to the variability of commercial and laboratory made extracts are due to the variability of stock cultures used to prepare allergenic extracts, the use of mycelial rich material as the source of allergens, the conditions under which molds are grown and extracts prepared, and finally to the stability of the proteins present in the extracts.

The allergenicity of an extract or fraction can be evaluated by prick testing or intradermal testing of allergic subjects. The most common *in vitro* tests for assay of allergen activity are RAST and ELISA (Kurup 1986; Turner et al. 1980). The RAST and ELISA can be adapted to obtain quantitative and semiquantitative estimations of antibody in the sera of patients. The allergenic activity and specificity of the allergen can also be compared by competitive inhibition assays. The stability of allergenic extracts depends on the chemical nature and quality of the allergen, the storage temperature, and the presence of preservatives and other nonallergic materials in the mixture.

4.1 Recombinant Allergens

Recombinant allergens are valuable tools to investigate the T-cell and B-cell involvement in allergic process as well as to study the pathogenesis of the disease (Scheiner and Kraft 1995). A number of recombinant fungal allergens from various sources with structural and biological properties comparable to the native counter parts have been reported (Valenta et al. 1997). The primary structure at the amino acid sequence level demonstrate extensive homology among the allergens and hence, it appears that the number of epitopes needed for diagnosis and specific therapy is less diverse than originally anticipated.

Specific immunotherapy for type I allergy should be based on reducing allergic reactions to those allergens, which are recognized by the patients' IgE antibodies. The establishment of patients' IgE reactivity profile (allergogram) with recombinant allergens will be of value in selecting the components against which a substantial IgE response is mounted. Selection of allergens according to the patients' allergogram might improve both diagnostic and therapeutic specificity of the allergens. Allergograms may help in monitoring the effect of treatment and immunotherapy (Hiller et al. 2002). The various allergens identified from the major fungi associated with allergy and approved by the International Allergen Nomenclature Committee of the International Union of Immunological Societies are shown

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Table 2 Mold allergens approved by the allergen nomenclatural committee[a]

Fungus	Mol. size (kDa)	Biological activity	Sequence accession number
A. alternata			
Alt a 1	28		U82633
Alt a 2	25		U62442
Alt a 3		Heat shock protein 70	U87807
			U87808
Alt a 4	57	Prot. disulfidisomerase	X84217
Alt a 6	11	Acid. ribosomal protein P2	X-78222
			U-87806
Alt a 7	22	YCP4 protein	X-78225
Alt a 10	53	Aldehyde dehydrongenase	X-78227
			P-42041
Alt a 11	45	Enolase	U82437
Alt a 12	11	Acid. ribosomal protein P1	X84216
C. herbarum			
Cla h 1	13		
Cla h 2	23		
Cla h 3	53	Aldehyde dehydrongenase	X-78228
Cla h 4	11	Acid. ribosomal protein P2	X-78223
Cla h 5	22	YCP4 protein	X-78224
Cla h 6	46	Enolase	X-78226
Cla h 12	11	Acid. ribosomal	X85180
A. flavus			
Asp fl 13	34	Alkaline serine protease	
A. fumigatus			
Asp f 1	18		M-83781
			S-39330
Asp f 2	37		U-56938
Asp f 3	19	Peroxisomal protein	U20722
Asp f 4	30		AJ001732
Asp f 5	42	Metalloproteinase	Z-30424
Asp f 6	26.5	Mn superoxide dismutase	U53561
Asp f 7	12		AJ-223315
Asp f 8	11	Ribosomal protein P2	AJ224333
Asp f 9	34		AJ223327
Asp f 10	34	Aspartic proteinase	X85092
Asp f 11	24	Peptidyl prolyl isomerase	
Asp f 12	90	Heat shock protein P90	
Asp f 13	34	Alkaline serine proteinase	
Asp f 15	16		AJ002026
Asp f 16	43		g3643813
Asp f 17			AJ224865
Asp f 18	34	Vacuolar serine proteinase	
Asp f 22w	46	Enolase	AF284645
A. niger			
Asp n 14	105	Beta-xylosidase	AF108944
Asp n 18	34	Vacuolar serine proteinase84b	
Asp n ?	85		Z84377
A. oryzae			
Asp o 13	34	Alkaline serine proteinase	X17561
Asp o 21	53	TAKA-amylase A	D00434, M33218
P. brevicompactum			
Pen b 13	33	Alkaline serine proteinase	
P. chrysogenum (formerly *P. notatum*)			
Pen ch 13	34	Alkaline serine proteinase	
Pen ch 18	32	Vacuolar serine proteinase	
Pen ch 20	68	N-acetyl glucosaminidase	
P. citrinum			
Pen c 3	18	Peroxisomal membrane protein	
Pen c 13	33	Alkaline serine proteinase	
Pen c 19	70	Heat shock protein P70	U64207
Pen c 22w	46	Enolase	AF254643
P. oxalicum			
Pen o 18	34	Vacuolar serine proteinase	

[a] http://www.allergen.org IUIS Allergen List.

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in Table 2 (IUMS—International Union of Immunological Societies 2002).

4.1.1 *Alternaria alternata*

Alternaria alternata, a member of the imperfect fungi, is one of the most important among all allergenic fungi (Achatz et al. 1995; De Vouge et al. 1996; 1998). Several allergens from *A. alternata* have been purified and characterized either by conventional fractionation or by employing molecular biology techniques (Table 2). Alt a 1, a major allergen of *Alternaria*, binds to IgE of more than 80% of asthmatic patients with allergy to this fungus. A number of variants and isoforms of Alt a 1 have been reported (Achatz et al. 1995; De Vouge et al. 1996). Alt a 1 with two subunits of 15 KD each, interact with IgE antibody only when present in unreduced form indicating the presence of conformational epitopes in Alt a 1 specific for IgE antibodies. Achatz et al. (1995) described three additional cDNA clones encoding functionally active *A. alternata* allergens. Alt a 7, the 22 KD allergen demonstrated over 70% sequence homology with the YCP4 protein of *Saccharomyces cerevisiae*, while Alt a 6 the 11 KD protein demonstrated homology with ribosomal P2 protein. However, these allergens reacted diversely with IgE from sera of allergic patients.

4.1.2 *Cladosporium herbarum*

Cladosporium herbarum is distributed widely in our environment and is a major source of inhalant allergens. *A. alternata* is the predominant fungus demonstrated in warm and humid climate, while *Cladosporium* is the leading allergenic mold observed in cooler climates. About 60 antigens from *C. herbarum* have been identified by crossed immunoelectrophoresis and about 36 of them have been shown to react with IgE from patients' sera by crossed radioimmunoelectrophoresis (Aukrust 1979). Three major *C. herbarum* allergens have been purified and characterized (Table 2). Cla h 1 is a small 13-KD acidic allergen composed of five isoallergens (pI 3.4–4.4), and Cla h 2 is a slightly larger molecule with a size of 23-KD is a glycoprotein (pI of 5.0) with over 80% carbohydrates (Sward-Nordmo et al. 1985; 1989). The protein part retained the IgE binding property even after removing the carbohydrate from this allergen. Cla h 3, the ribosomal P2 protein, is a low-molecular weight (11-KD) acidic allergen (pI 3.94) with high alanine and serine content, shares 60% sequence homology with ribosomal P2 protein (Zhang et al. 1995a,b). Breitenbach and his coworkers recently reported a purified recombinant *Cladosporium* enolase (Cla h 6; 48 KD) having strong binding to IgE antibodies in 20% of *Alternaria* allergic patients by immunoblots (Breitenbach et al. 1997). Enolase has been found to be a highly conserved major allergen in most fungi, and may contribute to allergen cross-reactivity in mold allergy. About 50% of the serum IgE from patients sensitized to *Alternaria* and *Cladosporium* showed binding to enolase. An allergenic HSP 70 has also been isolated from this organism (Zhang et al. 1995a,b).

4.1.3 *Aspergillus* Species

Aspergillus species are saprophytic fungi widely distributed in nature and are associated with a number of human diseases (Kurup and Apter 1998; Kurup and Kumar 1991). Several recombinant allergens from *A. fumigatus* have been identified and purified from cDNA and phage display library of *A. fumigatus* (Table 2). The majority of these proteins showed specific binding to IgE from asthmatic and allergic bronchopulmonary aspergillosis patients. Some of these *A. fumigatus* allergens also exhibited high sequence homologies with the known functional proteins and enzymes (Banerjee et al. 1998; Crameri 1998; Kurup and Banerjee 2000). Alkaline serine proteinases with allergenic properties such as Asp f 13, Asp f l 13, and Asp o 13 from *A. fumigatus*, *A. flavus*, and *A. oryzae* respectively have been reported (Shen et al. 1998). Similar serine proteinases Pen b 13, Pen c 13, and Pen n 13 with sequence homology to *Aspergillus* proteinase have also been identified from various species of *Penicillium* (Shen et al. 1997). Recently, another group of homologous vacuolar serine proteinases Asp f 18, Asp n 18, Pen n 18, and Pen o 18 with conserved sequence have been reported from *Aspergillus* and *Penicillium*.

Aspergillus flavus extracts demonstrated IgE antibody binding in 44% asthmatic patients studied by immunoblotting. Recently a 34 KD alkaline serine proteinase, Asp fl 13 with significant IgE antibody binding was cloned and the allergen expressed (Chou et al. 1999). The cDNA of this allergen Asp fI 13, showed 100% sequence identity to the 34 KD alkaline serine proteinase, major allergen Asp o 13 of *A. oryze*. Glucoamylase, cellulase, and hemicellulase from *A. niger* have been shown to be allergenic by skin prick tests and by *in vitro* specific IgE detection assays (Baur et al. 1988). In addition, a β-xylosidase (Asp n 14) from *A. niger* and α-amylase of *A. oryzae* has been recognized as an important occupational allergen in baker's asthma.

4.1.4 *Penicillium* Species

Among the environmental fungi, *Penicillium* and *Aspergillus* are the two dominant fungal genera frequently demonstrated indoors. Intradermal skin reactivity in atopic subjects using different mold extracts demonstrated 68% skin test reactivity to *P. notatum* (Burge 1985). Among the allergens of *Penicillium* species, the 32–34 KD alkaline serine proteinases were the major allergens of *P. citrinum*, *P. brevicompactum*, *P. notatum*, and *P. oxalicum* (Table 2). These allergens showed cross-reactivity with other major allergens from different *Penicillium* and *Aspergillus* species (Burge 1985).

4.1.5 Other Fungi

Aerobiological study performed in different countries demonstrated the presence of *Botrytis*, *Phoma*, *Helminthosporium*,

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Fusarium, and *Epicoccum*. In addition to these fungi, *Saccharomyces*, *Malassezia furfur*, *Candida albicans*, and Basidiomycetes spores including mushrooms and *Trichophyton* species that invade the skin have been implicated in allergic diseases (Baldo and Baker 1988; Horner et al. 1995; Ishiguro et al. 1992; Schmidt et al. 1997; Woodfolk et al. 1998).

5 IMMUNODIAGNOSIS

Demonstration of circulating antibodies against different *allergens* in the sera of sensitized subjects is an important criterion in the diagnosis of fungal allergy. A variety of tests with varied sensitivities have been developed to detect circulating antibodies to allergens from various sources. *In vivo* measurement of allergen specific IgE consists of skin prick test and intracutaneous test and both involved the production of wheal and erythema reaction, the characteristics of atopic sensitization. On the other hand, *in vitro* tests such as radioallergosorbent test (RAST), enzyme linked immunosorbent assay (ELISA), and Western blot, detect circulating antibody against specific allergens in sera from allergic patients (Kurup 1986; Turner et al. 1980). *In vitro* testing may be of use under special circumstances such as when patients are under medications, which interfere with skin testing and in some cases to avoid the possibility of anaphylaxis and uncomfortable local reaction. In addition, for patients with dermographism and wide spread skin diseases, *in vitro* IgE determination may be the better option for immunodiagnosis. ELISA has been demonstrated to be a sensitive, reliable, and semi-quantitative diagnostic tool for allergy. Although the sensitivity of the conventional ELISA system depends on many variables, the nature and type of antigens used and their ability to bind to polystyrene plates are the most important decisive factors (Kurup and Fink 2002).

Western blot involves the separation of antigens in polyacrylamide gel followed by transfer of separated proteins onto nitrocellulose membranes. The reactivity of these proteins with antibody in the sera of patients can be visualized on the membrane. Western blot has been used with great success in detecting specific antibodies to fungal antigens. Analysis of sera from patients suffering from allergic asthma, hypersensitivity pneumonitis due to *A. fumigatus* and ABPA by Western blot showed heterogeneous IgG subclasses and IgE responses (Kurup et al. 1994).

Radioimmunoassay (RIA) was developed to measure antibodies to radiolabeled acid-soluble antigenic fractions of various allergenic extracts. Radioallergosorbent test, the most commonly used RIA, has extensive application in the detection of IgE class to various allergens (Yunginger et al. 1976).

5.1 Semiautomated Specific IgE Assay (Pharmacia ImmunoCAP System)

The Pharmacia ImmunoCAP system is a semiautomated system for the measurement of circulating IgE antibodies. This assay is calibrated against the World Health Organization standard for IgE and allows quantitative expression of total or allergen specific IgE. The results are expressed as kilo units of allergen specific IgE per liter (KU_A/l). In an effort to establish recombinant allergens as standardized reagents for the diagnosis of ABPA, the ImmunoCAP system being investigated using recombinant allergens from *A. fumigatus*. The results obtained were comparable to that of ELISA. Although recombinant allergens from *A. fumigatus* and other fungi have shown great promise as suitable allergens for the development of a fully automated diagnosis of fungal allergy has not yet been developed. Before selecting allergens, the panel of fungal allergens must be individually evaluated using ImmunoCAP system. It is also essential to standardize the test for reliability and sensitivity (Hemmann et al. 1998).

5.2 Skin Test

Two types of skin reactions can be observed in allergic patients. Type I (immediate) reactivity consists of a "wheal and flare" within 5–20 min of antigen challenge. The type III reaction (Arthus reaction) is an induration usually developed within 4–8 h after skin testing. The reaction usually subsides after 24 h, but occasionally a delayed reaction persists for 24 h or more. Patients are regarded as being sensitive to a particular antigen preparation if the immediately developing wheal diameter is greater than 4 mm. Until recently crude fungal antigens have been used for skin testing patients, however, the results of these reactions are not always satisfactory. In recent years, purified recombinant allergens from *Aspergillus*, *Alternaria*, and *Cladosporium* have been used with considerable success. The significance of the late skin response is not yet fully understood, but may be associated with the infiltration of eosinophils in pulmonary diseases such as ABPA.

6 IMMUNOTHERAPY AND VACCINATION IN MOLD ALLERGY

Allergen specific therapy appears feasible in the future because of the availability of an increasing number of functional recombinant fungal allergens. Allergen specific therapy may aim at the prophylaxis of atopy through induction of tolerance or modification of ongoing immune reactions. Such attempts may be based on the application of T-cell epitopes as multiple peptides, pure recombinant allergens, or as IgE binding haptens. More recent approaches such as modification of allergens by manipulating allergen encoding cDNAs (i.e., site directed mutagenesis) or gene immunotherapy using cDNA encoding relevant allergens (naked DNA therapy) and modulation of immune responses using immunostimulatory sequences such as CpG. However, further studies are essential in this field to understand the effectiveness and mechanisms. In addition, isoforms of major allergens and synthetic peptides with very low IgE binding

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capacity, but possessing strong T-cell stimulating sequences may be explored for inducing immune deviation from a Th2 to Th1 cytokine pattern to reduce or alleviate the allergic response.

7 CONCLUSIONS

There has been significant progress in fungal-induced allergy, particularly with regard to the development of diagnostic assays and in the production of well-characterized allergens. With the advent of these well-characterized allergens either purified via conventional methods or through molecular engineering technique contribute to improved specific diagnosis of the disease. By understanding the specific roles of various epitopes of these allergens, in stimulating T- and B-cells, may eventually provide insight into the immunopathogenesis of the disease. The secretory products such as cytokines and chemokines resulting from such activation may open up avenues for targeted therapy to control or treat the disease. Effective immunotherapeutic and vaccination regimens may result from such understanding on the structure function interaction of the allergens and the immune system.

Asthma therapy like other treatments is currently moving towards a molecular regimen. There are several preclinical studies utilizing DNA to address asthma and allergy therapeutics. These include use of antisense oligonucleotide against nuclear transcription factors such as GATA-3 gene transfer using both plasmids and viral vectors and CpG oligodeoxy nucleotides. These innovative measures may yield successful immunomodulatory therapy for asthma and atopic diseases.

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Current Status of Fungal Collections and Their Role in Biotechnology

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1 INTRODUCTION

The vast array of uses and properties described in this handbook is a testimony to the countless ways in which mankind has harnessed fungi. Characterization techniques have been developed continuously in recent years and new properties are being discovered at a rapid pace. Therefore, it is essential that methods for maintenance and preservation must be optimized to ensure that strains retain their full potential. The removal of an organism from its natural environment, and the subsequent manipulation that occurs through maintenance on synthetic media, while maintaining viability, may induce selection from the original population of cells isolated. Consequently, preservation of a living strain for long periods may lead to irreversible loss of properties. Thus, fungal culture collections must not only keep the organisms viable but also must ensure that they retain their full genetic and physiological integrity. Collections of organisms are not new; records of them being kept by individuals in public and private organizations date back to the 1800s (Sly and Kirsop 1990). There continues to be a demand for living collections and their specialist services but there is also a need for them to be better integrated and networked internationally to improve coverage and increase access.

1.1 The Biological Resource Center

Culture Collections have become much more than organism repositories and many are Biological Resource Centers (BRCs) being generators of information and sources of expertise.

An OECD BRC Initiative was established in 1999 to try and secure the future of microbial resource collections. Since then the definition of a BRC has broadened to include a wider range of organisms. A report on the first phase was published in March 2001 *Biological Resource Centres Underpinning the future of life sciences and biotechnology* that made a call for action by OECD countries and beyond (OECD 2001). The report recommends that governments, the scientific community, and the private sector work together to achieve five goals:

a. Establish national BRCs
   Selectively seek to strengthen existing *ex situ* collections of biological data and materials and enhance their quality to a level required for accreditation as national BRCs. In addition, when needed, create new collections, including countries outside the OECD.
b. Develop an accreditation system for BRCs based on international criteria
   Support the development of an accreditation system for BRCs based upon scientifically acceptable objective international criteria for quality, expertise, and financial stability.
c. Create international linkages among BRCs
   Facilitate international co-ordination among national BRCs by creating an agreed system of linkage.
d. Co-ordinate standards, rules, and regulations taking BRCs into account

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Take into account the objectives and functioning of BRCs when establishing and harmonizing national or international rules and regulations.

e. Establish a global BRC network
Support the establishment of a global BRC network that would enhance access to BRCs and foster international co-operation and economic development.

These are essential elements that must be drawn together and implemented if comprehensive coverage of microbial diversity to appropriate quality standards is to be achieved. If BRCs are to work together they need to operate to common standards and must be able to retain the full potential of the organisms they hold. Making data available on new species and their properties will help in the search for microbial solutions to agricultural, economic, environmental, food, and public health problems. This chapter will describe the current status of fungal culture collections, and how they are adapting their products and activities to the changing needs of the twenty-first century and making progress toward the OECD recommendations.

2 CULTURE COLLECTIONS: FORM AND FUNCTION

2.1 Background

Public service culture collections are charged with several tasks that underpin life sciences and provide tools for biotechnology. Collections are custodians of *ex situ* genetic resources and have a key role to play in their conservation (Kirsop and Hawksworth 1994) helping; countries meet their obligations under the terms of the Convention on Biological Diversity (CBD). The main functions of public service collections include:

a. The *ex situ* conservation of organisms.
b. Custodians of national resources.
c. Provision of living resources to underpin the science base.
d. Receipt of deposits subject to publication.
e. Safe, confidential, and patent deposit services.

2.1.1 The Role of the Modern Biological Resource Center

The form and function of traditional Culture Collections have been adapted over the years to enable them to meet the changing requirements of the scientific community and national governments. There are many different types of collection, each having the common theme to conserve micro-organisms and support life sciences but with many additional and different objectives. Living collections are maintained to provide reference points for names, vouchers for research and patents, production strains, and organisms for screening and research. The types of microbial resource collections range from small private collections through to large service collections that may have widely differing policies and holdings. Collections can be established to keep strains required for a specific scientific study through to ones built up over many years for private reference or research. Similarly, small, specialized collections can be established to support an industrial activity, for e.g., a microbial resistance test house may maintain a collection of strains cited in test specifications. Collections can also be established to supply cultures for specified purposes, for e.g., in teaching or research. Some collections are specifically tasked to be repositories for "type" cultures, name-bearing strains that fix the application of names. Living types are required for bacteria, and cultures preserved in a metabolically inactive state have been approved for fungi since 1993. Most of these collections have specific remits and maintain a finite selection of organisms. Service collections can have a wider remit and may collect an array of organisms that are considered to be of interest to the wider scientific community. However, the conservation of microbial diversity *per se* is rarely the main objective of a collection.

The investment in a large broad spectrum collection, in terms of facilities, personnel, and expertise is substantial and a cost-effective rationale is required for its maintenance and long-term support. Worldwide, no collection is able to totally finance its activities purely on strain sales alone. Indeed, of the 470 plus collections listed in the *World Directory* (Sugawara et al. 1999) almost 400 are government owned or sponsored and dedicated to operations related to the home institute's activities. For e.g., the provision of diagnostic services, the search for exploitable metabolites or enzymes, the direct use of organisms as food, or food modification, as biocontrol agents, or as biodegraders or bioremediators. Although, there is still significant government support for many collections worldwide, there has been a dramatic trend in the disappearance of government-funded culture collections. A comparison of statistics published on the World Data Centre for Microorganisms (WDCM) web site (http://wdcm.nig.ac.jp/statistics2000.html) shows that 17 collections were lost between June 2000 and August 2001, 13 of which were government-sponsored. Despite this, there was an overall increase in holdings from just over 900,000 to 1.06 million, a total of 141780 more strains being maintained.

Biological Resource Centers are under pressure to be self-sustaining, many are moving toward this by being more selective in what they collect, by adding value to their holdings through characterization and screening programs and by getting more involved in commercial utilization of their products. Although this trend may help secure them for the long term what happens to the conservation and comprehensive support they provide to research and education? A balance is being sought, the OECD BRC Initiative may provide some answers.

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2.1.2 The Potential *Ex Situ* Resource

The search for organisms with unique properties continues. Bioprospecting, geared towards discovering novel properties and products from living organisms is cyclical but has been intensive. Concurrent to this development is the discovery of new micro-organisms. It is essential that representatives of these and other useful organisms are maintained for future use. If a strain is lost, recovery of that strain or even a strain of the same species from its natural environment can be difficult or for practical purposes impossible unless it is a common organism and has a specific host or environment from a habitat that is not under threat from destruction. Even if the same species is re-isolated, the properties of the strain may be different due to the considerable intraspecific variation that exists. Humans can have a devastating effect on the environment and habitat destruction will eventually destroy a proportion of the microbial diversity. The *ex situ* conservation of micro-organisms from the environment can ensure their availability for future use if their natural environment is destroyed. It is therefore imperative that isolates are adequately preserved to maintain their integrity and for future use for re-introduction into reclaimed land, in screening, genetic improvement, characterization, and the production of desirable end products.

Microbial resource collections have developed on an *ad hoc* basis and are not considered capable of adequately conserving this vital world resource (Allard et al. 1993). For fungi alone, there is an estimated 1.5 million species throughout the world, of which only 74,000 have been described (Hawksworth 2001), of these, only 11,500 ($<1\%$) are held in the world's culture collections. Each year over 1000 new species of fungi are described and catalogued in the *Index of Fungi* (Hawksworth 2001), at this rate, it will take the world's mycologists 1426 years to discover and describe all species of fungi. To store representatives of these, it would require the 250 fungal collections registered with the World Data Centre for Microorganisms to store 6000 species each. However, it is important to ensure that an adequate and appropriate representation of the variability of each species is taken into consideration and this would require many more fungi to be stored. There is not one strain of the majority of these species that can represent the full spectrum of morphology and physiology expressed within that species and therefore there is a requirement to retain a range of representative strains. The CABI Bioscience Genetic Resource Collection holds on an average 5 strains per species, but this is considered woefully inadequate in many cases. For e.g., when considering host specificity in the plant pathogen *Fusarium oxysporum*, which has over 120 special forms (Kirk et al. 2001). The problem is further exaggerated when fungal genomics are considered, for e.g., *Aspergillus nidulans* is known to have some 13,000 genes (Kirk et al. 2001), so the number of potential genetic variants is considerable. A conservative estimate of the number of strains to represent each species could be between 10 and 50 strains, substantially increasing the number of strains that would need to be housed in the world's 250 fungal culture collections. To make any impression on such an enormous task there must be a focus for each collection and a sharing of tasks between them. The task of maintaining representative collections of fungi should not be left to the current *ad hoc* system. There are many countries particularly rich in biological diversity having a wide range of ecosystems yet to be fully explored. The task of conservation necessitates the setting of priorities in order to make inroads in a logical way. It has been suggested that priority be given to the conservation of useful organisms (Wood 1992), although it is difficult to predict which may be useful in the future.

2.1.3 Access to the Information Resource

The information explosion and access to it via the Internet can lead the microbiologist to a plethora of information relating to biological resources. An increasing number of collections, microbiology laboratories, and biotechnology companies provide information via the Internet (Table 1). Yet, it is still convenient to have texts and catalogs available, so that information can be rapidly accessed when needed. The UKNCC provides information on its website http://www.ukncc.co.uk but has also published a Biological Resource book that provides the information needed to isolate, grow, preserve, and characterize micro-organisms (Smith et al. 2001). Such information provided by collection organizations is vital to ensure microbiologists are operating to the best available procedures and within regulatory frameworks when preserving and distributing their organisms.

2.1.4 Control of Access Through the Convention on Biological Diversity

In recognition of the continued depletion of biological resources and the need for conservation of the world's biodiversity, the Convention on Biological Diversity was agreed at the Earth Summit in Rio de Janeiro in 1992. The Convention came into force in December 1993 and has now been ratified by more than 180 countries. The aim is to secure the genetic pool and to enable its sustainable utilization whilst ensuring equitable sharing of accrued benefits. Sovereign rights over genetic resources are assigned to the country of origin and countries are required to facilitate access to them through biodiversity inventories. The CBD requires that national strategies for the conservation and sustainable use of biological diversity are developed. These should enable the identification, sampling, maintenance of species and their habitats, and the production of inventories of indigenous species.

Biological Resource Centers play an important part in these processes. The CBD requires that Prior Informed Consent (PIC) be obtained in the country where organisms are to be collected before access is granted. Terms, on which any benefits will be shared, must be agreed in advance. The benefit sharing may include monetary elements but may also include information, technology transfer, and training.

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Table 1 Website links to cited organizations

Topic	Organization	Web link
Collection organizations	Belgian Co-ordinated Collections of Microorganisms (BCCM)	http://www.bccm.belspo.be
	CABI Bioscience UK Centre	http://www.cabi-bioscience.org
	Common Access to Biological Information (CABRI)	http://www.cabri.org
	European Culture Collection Organisation (ECCO)	http://www.eccosite.org/
	UK National Culture Collection (UKNCC)	http://www.ukncc.co.uk
	World Data Centre for Microorganisms (WDCM)	http://wdcm.nig.ac.jp/
	World Federation for Culture Collections (WFCC)	http://www.wfcc.info/
Convention on biological diversity	Convention on Biological Diversity	http://www.biodiv.org/
	International Code of Conduct—Belgian Co-ordinated Collections of Microorganisms	http://www.belspo.be/bccm/mosaicc/
Patents	World Intellectual Property Organisation (WIPO)	http://www.wipo.org/
Packaging and shipping micro-organisms	International Air Transport Association	www.IATA.org/cargo/dg
	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH	http://www.gbf.de/dsmz/shipping/shipping.htm
Quality guidelines	Common Access to Biological Resources Information	http://www.cabri.org/guidelines/gl-framed.html
	UK National Culture Collection	http://www.ukncc.co.uk/html/Information/docs/UKNCCQAP.doc
	World Federation for Culture Collections	http://wdcm.nig.ac.jp/wfcc/GuideFinal.html

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If the organism is passed to a third party it must be under terms agreed by the country of origin. This will entail the use of material transfer agreements between supplier and recipient to ensure benefit sharing with, at least, the country of origin. Many BRCs have operated benefit sharing agreements since they began, giving organisms in exchange for deposits and re-supplying the depositor with the strain if a replacement is required. An EU DG XII project, Microorganisms Sustainable Use and Access Regulation International Code of Conduct (MOSAICC) has published procedures to allow traceability and enable compliance with the spirit of the CBD and with national and international laws governing the distribution of micro-organisms, whilst not restricting scientific goals (Davison et al. 1998). The development of such common procedures is an evolutionary process and the co-ordinators of this project have placed the document on their web site and amend it as it develops (http://www.bccm.belspo.be). The BRCs offer transparency to the implementation and operation of the CBD.

2.1.5 Protecting Intellectual Property Generated from Micro-Organisms

Novel micro-organisms with unique properties have been included in patent applications for the protection of the intellectual property rights of a researcher and institution (Kelley and Smith 1997). The CBD does not prevent this procedure, although sovereign rights over genetic resources are assigned to the country of origin, but this does not preclude others owning Intellectual Property associated with them as long as the country of origin has an equitable share in benefits. One major requirement for granting a patent is the deposition of the strain in an International Depository Authority (IDA) under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure http://www.cnpat.com/worldlaw/treaty/budapest_en.htm. There are currently 29 IDAs in the world (World Intellectual Property Office 1996)—an increased level of local bioprospecting will lead to more (novel) micro-organisms with novel applications being discovered, thus, increasing the demands on IDAs. To fulfill the requirements of the Budapest Treaty and to be recognized as an IDA, a microbial resource collection must meet a number of quality requirements.

a. Located in an approved contracting state.
b. Have a continuous existence.
c. Have the necessary staff and facilities.
d. Be impartial and objective.
e. Be available to any depositor on the same basis.
f. Accept specified kinds of organisms, examine their viability, and store them as prescribed in the regulations for 30 years.
g. Issue receipt and viability statements.
h. Comply with confidentiality agreements.
i. Furnish samples in conformity with the regulations.

2.2 Mycological Collections

Although most public culture collections are tasked with the preservation and maintenance of a wide range of micro-organisms and cell lines, a number of major international collections have significant collections of fungi (Table 2). The World Data Centre for Microorganisms (WDCM) provides information on the collections registered with the World Federation for Culture Collections (WFCC) and can be found at http://wdcm.nig.ac.jp. Statistics provided on the website are updated periodically and they currently report that the 466 collections registered from 61 countries hold over 1.06 million strains of which almost 350,000 are fungi. Over half, around 250, of the registered collections hold fungi. There are 23,000 fungal names listed but this fails to take into account the anamorph/teleomorph linkages and synonyms and therefore, there are considerably less species represented. Many

Table 2 Some major international culture collections that have significant fungal holdings

Collection and location	Acronym	URL	Approx. no. of strains
Agricultural Research Service Culture Collection	NRRL	http://nrrl.ncaur.usda.gov	45,000
American Type Culture Collection, USA	ATCC	http://www.atcc.org	27,000
CABI Bioscience, UK (previously, International Mycological Institute)	IMI	http//cabi-bioscience.org	23,000
Canadian Collection of Fungal Cultures	CCFC	http://res.agr.ca/brd/ccc/ccctitle.html	10,000
Centraalbureau voor Schimmelcultures	CBS	http://www.cbs.knaw.nl	32,000
Food Science Australia, Ryde	FRR	—	4000
Fungal Genetic Stock Centre, USA	FGSC	http://fgsc.net	14,000
Institute of Fermentation, Osaka	IFO	http://www.ifo.or.jp/index ehtml	7768
Mycothèque de l'Universite Catholique Louvain	MUCL	http://belspo.be/bccm	25,000
University of Alberta, Microfungus Collection and herbarium	UAMH	http://www.devonian.ualberta.ca/uamh/	9633

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collections contain representatives of fungi that are commonly collected and can be easily preserved. However, increasingly collections are developing protocols targeted towards preservation of recalcitrant fungi that are less common, but are of immense scientific importance.

2.3 Operations Quality Management

It is imperative that organisms utilized in biotechnology are maintained in a way that will ensure that they retain their full capability. Biological Resource Centers must ensure a quality product providing standard reference material that will give reproducible results. To achieve this, collections must apply quality control and assurance measures to maintain these standards, taking into account the needs of users and of the facilities and resources available. The need for common standards is evident as the task of maintaining representative samples of microbial diversity cannot be achieved by one collection alone. Therefore, it is essential that a world-wide network of collections interacts to provide the coverage required by the user. In order that a customer of such a network would get a consistent level of service and quality it is necessary to set standards for all collections to attain. These standards would also provide a useful target for new collections to achieve. Although there are guidelines set for the establishment and operation of collections (http://wdcm.nig.ac.jp/wfcc/index.html), they do not cover all protocols or procedures, nor do they set minimum requirements. Standards are necessary to maintain quality in collections and to ensure they offer the service to science and industry that is required today and provide stable reference material for the future. Examples of existing standards for collections are:

a. The WFCC Guidelines for the establishment and operation of collections of micro-organisms (http://wdcm.nig.ac.jp/wfcc/index.html).
b. The Microbial Information Network for Europe (MINE) project standards for the member collections (Hawksworth and Schipper 1989).
c. UKNCC quality management system (http://www.ukncc.co.uk).
d. Common Access to Biological Resources and Information (CABRI) guidelines (http://www.cabri.org).

There are also standards, which can be applied to microbiology laboratories such as Good Laboratory Practice (GLP), ISO 17025, ISO Guide 25, and ISO 9000 series. Industry is expressing the need for quality control and standards within collections. Although publications on collection management and methodology give information on protocols and procedures (Hawksworth and Kirsop 1988a,b; Kirsop and Kurtzman 1988; Kirsop and Doyle 1991; Smith and Onions 1994) the UKNCC quality management system goes further toward setting minimum standards. There are critical elements in the handling, storage, characterization, and distribution of micro-organisms and cell lines and to the handling of associated information. Setting required minimal and preferred standards for these elements will ensure reproducibility.

2.3.1 Identified, Authenticated Strains

The identification and authentification of collected strains is essential. Holding material without descriptive data will cause unnecessary duplication and will waste resources. If the organism cannot be named then descriptive data, photomicrographs, metabolic profiles, and sequencing data are extremely useful. Collections must store characterized strains and the methods used to record such data must be standardized to allow optimized transfer of information should it be required. A published system for collection databases is the Microbial Information Network Europe database (Gams et al. 1988; Stalpers et al. 1990).

2.3.2 Purity, Viability, and Stability of Strain Properties

The purity and viability of strains should be checked and recorded before and immediately after preservation and periodically during storage. Strains should be pure wherever possible, although there are exceptions where strains cannot be grown without their symbiont, host, or food organisms. However, it is imperative that such requirements are recorded and defined. Viability testing programs varies with the methods used, but the data obtained should demonstrate if a strain is deteriorating during storage. Acceptable viability for many fungi is the germination and subsequent development of in excess of 75% of propagules/cells, although an acceptable level may be set at 50% or possibly lower for some cell types. Any deviation from set standards would need to be explained and recorded. A program of tests to ensure stability of strains must be put in place. Known properties can be checked periodically but full metabolic profile checks are seldom necessary on a regular basis.

2.3.3 Methodology and Equipment

Wherever possible, optimized techniques and standard procedures should be adhered to. It is necessary that all procedures are fully documented, so that all staff can repeat them accurately. This includes all measuring and recording techniques from viability and purity checks through preservation methods to characterization and the checking of properties. The accepted level of deviation from measurable parameters must be set and records maintained to show that performance is within the accepted limits. Documented procedures are essential to ensure continuity and new staff must be trained to ensure the attainment of the standards set. Training must include method performance, an understanding of expected levels of results and variation

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tolerances, monitoring and recording. All equipment used in the BRC must be regularly maintained and calibrated and must operate to set limits within international standards. All details must be recorded to ensure traceability and reproducibility.

2.3.4 Long-Term Security

There is little point in establishing a collection without considering the long-term security of its holdings. If procedures are put in place that cannot be maintained in the future, then a considerable waste of time and resources is inevitable, for e.g., freeze-dried collections need little maintenance but frozen collections must be kept cold at a designated temperature at all times. It is also good practice to ensure that there are "back-ups" of each culture, organisms should be stored by a minimum of two techniques, and a source of working and security stocks should be maintained. Further, all "important" strains and the information concerning them should be stored at another site as a "disaster" measure.

2.3.5 Auditing/Monitoring

It is essential that adherence to set standards is monitored at every level. The appointment of auditors from other departments, or even from outside the organization (required for many accreditation schemes), is beneficial. The recording of such monitoring is vital to demonstrate competence and self-checks should also be part of a good management system.

2.3.6 Legislation

There are many regulations that apply to the work of collections from the collecting, through handling to their dispatch and transport (Smith et al. 1999). Collection workers must be aware of such legal requirements not only in their own countries but internationally. Examples of the areas covered by regulations include packaging, shipping, and transport; quarantine, health and safety, patenting, and access to national genetic resources.

It is becoming more common for a collection to be asked what accreditation scheme or protocols they follow to ensure quality control of the product. High standards are required to meet enhanced customer expectations. At the very least, this requires set methods and levels of acceptability, recording of results and an independent monitoring system to enable the long-term security and sustainability of holdings. The UKNCC and CABRI systems referred to above provide an excellent framework.

3 PRESERVING GENOMIC INTEGRITY

Fungi are ubiquitous and can utilize many natural and synthetic substrates. A proportion are host specific while others have unknown growth requirements and cannot be maintained *in vitro*. However, most fungi will thrive on media that contain extracts of natural materials, similar to those in the environment from which they were isolated. Many fungi produce propagules (e.g., spores, condia, sclerotia, and thickened hyphae), which allow them to tolerate unfavorable conditions; these can be targeted for preservation to help ensure long-term viability (Smith and Onions 1994). Nevertheless, some storage protocols allow uncontrolled growth and reproduction to occur, which may induce the organism to change and adapt to artificial laboratory conditions.

The aim of preservation is to maintain purity, viability and genomic integrity, avoid selection of variants from within a population, and lessen the prospects of strain deterioration. There are many preservation protocols suitable for fungi, but an array of criteria should be considered before preservation (Ryan et al. 2000) as no individual preservation technique has been successfully applied to all fungi. Storage in liquid nitrogen appears to approach the ideal and most fungi (even nonsporulating isolates) that grow well in culture survive cryopreservation in liquid nitrogen. However, Ryan et al. (2001), suggest that changes in physiology and genetic stability may occur in some isolates requiring optimal preservation protocols to be designed (Smith and Thomas 1998). For organisms that cannot be cryopreserved, other techniques may be appropriate, i.e., storage in soil, water or mineral oil, but the length of storage may be diminutive for some of these methods (Smith and Onions 1994).

3.1 Basic Methods

Storage under oil, water storage, silica gel storage, and soil storage are adequate, economical methods of preserving fungi (Table 3) although there may be pitfalls. Although, initial preservation is not very labor intensive compared with freeze-drying and cryopreservation, samples preserved by these methods will need to be sub-cultured at relatively frequent intervals to ensure that organisms remain viable and physiologically stable in the longer-term. For e.g., organisms preserved by water storage will require removal from storage, re-growth, and re-preservation after approximately 2 years (Smith and Onions 1994). These methods should only be used if other methods of preservation (i.e., cryopreservation, lyophilization) are not available because strain drift and contamination may compromise long-term integrity (Smith et al. 2001).

3.2 Freeze-Drying

The technique of freeze-drying (lyophilization) has been successfully applied to sporulating filamentous fungi for many years and is widely used for fungi by all of the major international culture collections. The basic technique involves slow cooling of samples followed by slow drying from the frozen state under vacuum. The use of modern equipment

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Table 3 Basic preservation methods

Method	Suitable for:	What is preserved?	Length of storage	References
Water	Most fungi	Agar Plugs	2 years (max)	Burdsall (1994); Smith et al. (2001)
Oil	Most fungi	Slope culture	Depending on species Oomycetes 12 to 13 months. Basidiomycetes 2 years	Fennell (1960); Smith et al. (2001)
Silica gel	Sporulating fungi	Spores	Up to 25 years	Perkins (1962); Smith et al. (2001)
Soil	Some sporulating fungi (e.g, *Fusarium* spp.)	Spores	Up to 10 years	Booth (1971); Smith et al. (2001)
Serial transfer[a]	Most culturable fungi	Inocula of spores or mycelium, typically as agar plug	Depending on species (fast growing isolates: 1 to 2 months, slower growing isolates as necessary)	Smith and Onions (1994); Smith et al. (2001)

[a] A maintenance method.

allows the process to be tightly controlled, which improves results and reproducibility. The residual moisture content should be below 2% but not less than 1% (Smith and Onions 1994). Freeze-drying is most suitable for members of the Ascomycota, Zygomycota, and some Basidimomycota and their anamorphs, with survival of in excess of 30 years for some isolates. However, freeze-drying is not suitable for all fungi, especially those that do not readily sporulate in culture, although some workers report that hyphae of some fungi can withstand freeze-drying (Tan 1997).

The main advantages of freeze-drying as a preservation method are that specimens are stored in air-tight glass ampoules, which prevents contamination during storage with very good long-term viability and stability for some fungi. Ampoules can also be easily distributed in UN-approved containers to customers through the postal services in an "inactivated" state, which reduces the prospects of strain damage occurring during transit. The main disadvantages of freeze-drying are that it is not suitable for all fungi (no single preservation method is), "lyoinjury" can occur during the cooling and drying stage (Tan 1997) and genetic damage may occur (Ashwood-Smith and Grant 1976; Ryan et al. 2001) and there may be delayed recovery after storage. The actual lyophil process is time-consuming, complex, and expensive, which may be prohibitive to smaller collections with little resources.

3.3 Cryopreservation

Cryopreservation of fungi in or above liquid nitrogen at temperatures of −140°C or below is the preferred method for long-term storage of biotechnologically important fungi. Cryopreserved cultures can be kept stable for relatively long periods as little metabolic activity occurs below −70°C, however, ice can re-crystallize at temperatures above −139°C, which can cause damage to fungi during storage. Damage or "cryoinjury" can also occur during the cooling and thawing stages of the cryopreservation procedure. Intracellular ice can physically damage cellular components and the osmotic effects that occur during ice formation and thawing can induce "concentration" effects (Merryman et al. 1977) causing membrane damage and disrupting the biochemical integrity of cells. Cryopreservation methods can be optimized for individual genera through the application of chemical cryoprotectants in association with controlled rate cooling regimes (see section 3.4 in this chapter). Cryopreservation is suitable for most fungal cell types, including hyphae, although viability may be poor for some cultures that do not readily sporulate in culture. As with all preservation methods, there are some disadvantages of cryopreservation. A major concern is the financial cost of establishing a cryopreserved collection, the equipment required to prepare and house the collection is expensive. A constant and reliable supply of liquid nitrogen is essential, as any interruption in supply could be disastrous. However, the advantages far out-weigh the disadvantages if resources are available because most fungi

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will survive the cryopreservation process, the preserved cultures are extremely stable and the potential length of storage is virtually limitless.

3.4 Protocol Development

3.4.1 Standard Approaches to Preservation Optimization

Although the majority of fungi can be adequately preserved using traditional preservation methods, some cannot and these are collectively termed "preservation recalcitrant fungi." Preservation recalcitrant fungi often include those which do not readily sporulate in culture, for e.g., members of the Oomycota (*Saprolegnia* spp., *Aphanomyces* spp.), some Basidiomycota (*Serpula lacrymans*) and others which are difficult to maintain in culture (e.g., *Diplocarpon*) or are facultative pathogens. Increasingly, protocol development is becoming essential for fungi of biotechnological importance as genomic and physiological instability has been reported in a number of fungi after preservation (Gaylarde and Kelley 1995; Ryan et al. 2001). If instability was to occur in fungi of economic importance, the consequences could be disastrous. For e.g., a change in the DNA fingerprint of a patent could result in legal complications; alternatively changes in the physiological integrity of a manufacturing isolate could result in significant financial loss. Therefore, it is a good practice for biotechnology companies to preserve their important organisms using optimized preservation protocols.

There are two approaches that can be employed to optimize a cryopreservation protocol. The first is to directly observe the effects of cooling and thawing on the test organism using a light cryomicroscope. The second is to compare the effects of defined preservation regimes on the viability, pathogenicity and morphological, physiological and genomic stability of replicates before and after preservation.

Cryomicroscopes have been used to examine the physical effects of cooling and thawing for many years (Smith and Thomas 1998). The cryomicroscope consists of a standard light microscope with a specially constructed stage, which is cooled by a continuous stream of cold nitrogen gas. A slide with an integral thermocouple is placed on the stage, which allows temperature to be controlled through a computer interface. Specifically designed cooling and thawing regimes are then programmed into the computer. Each replicate is tested with each regime and images are recorded for future analysis. The criteria for analysis include cell shrinkage, extracellular ice formation, intracellular ice formation, membrane disorganization, and cell rupture. The regime that causes the least apparent damage to the fungus is then tested using a controlled-rate cooler.

An alternative approach is to design experiments that assess the characteristics of a fungus before and after cryopreservation. A suite of characters is determined before preservation, these could be genetic fingerprints based on PCR techniques [i.e., Random Amplified Polymorphic DNA (RAPD), Arbitrary Length Fragment Length Polymorphism (AFLPs), Single Sequence Repeat (SSR)], secondary metabolite profiling (using HPLC/TLC) or enzyme assays, culture morphology and anatomy. The fungus is preserved using a number of different cooling regimes in the controlled-rate cooler. After thawing, each replicate is grown under the same conditions that were used to establish the suite of characters before preservation. Post preservation results are compared with those obtained before preservation and the regime that best maintains the stability of characters can be used for full-scale preservation.

3.4.2 New Approaches

In recent years, technology used to preserve other types of organisms, especially plant cells, has been transferred for use with fungi, especially those that are preservation recalcitrant. Two main approaches have been investigated, immobilization (encapsulation) and vitrification. Immobilization of fungi in calcium alginate beads was originally developed by the biotechnology industry to produce enzymes and other novel products (Kwak and Rhee 1992). It has also been used as a carrier for fungal products such as bioherbicides (Walker and Connick 1983) and ectomycorrhizal fungi (Mauperin et al. 1987). Immobilization has been extensively used for the preservation of plant cells (Benson et al. 1996) and a basic method has been applied in the preservation of the basidiomycete *Serpula lacrymans* (Ryan 2001a). Immobilization in calcium alginate allows cells to be manipulated. They can be processed in a relatively easy manner and prepared for preservation. Beads can be dehydrated, either osmotically or in a stream of sterile air, prior to preservation, which will reduce water content and lessen the prospects of cryoinjury. The potential of this technique for the cryopreservation of recalcitrant fungi is currently being investigated in a number of major culture collections including CABI Bioscience. The use of vitrification for the preservation of fungi is a recent development. Widely used for other cell types, e.g., plant cells (Benson 1994), the technique involves the addition of highly concentrated cryoprotectant solutions to mycelium and spore suspensions, the solution is allowed to penetrate the material, before cells are plunge-cooled in liquid nitrogen at $-196°C$. Vitrification solutions typically contain osmotically active compounds such as ethylene glycol and dimethyl sulfoxide, which have the potential to be extremely toxic. A protocol, originally designed for the vitrification and cryopreservation of plant cells, has been applied to a number of basidiomycetes with some success (Ryan, unpublished results). Vitrification has the potential to be used with a wide-range of preservation recalcitrant fungi.

3.5 Characterization

Fungal culture collections contain unique and interesting organisms, many of which have not been fully examined.

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Many of these are likely to have properties of interest to the biotechnology industry. Unlocking the value in culture collections is the challenge facing both the collections and the scientific community, which they serve. To detect potentially valuable isolates, collections must embrace evolving technologies used in the fields of functional genomics and phenomics, for e.g., microarray, which will allow high-throughput screening; although useful, traditional methods of characterization are often time-consuming and laborious. Collections already hold many organisms of importance, but will no-doubt contain countless more isolates in their holdings with potential for bioremediation and biodegredation; enzyme, metabolite and novel product production and as biological control agents (Ryan 2001b).

Even without sophisticated equipment, there are several simple media-based tests that can be used to help characterize fungi, detecting the presence of metabolites or enzymes or that can assess the ability to utilize specific compounds or materials. Building up a database of such information can help the search for biotechnological solutions. However, there are many more sophisticated biochemical and molecular methods that can provide such information (see Table 4).

Table 4 Characterization techniques used by BRCs to add value and help describe strains

Characterization	Test method(s)	Output
Enzymes	Media based—sole carbon sources etc. see below	List of enzyme producers for use in industry
	APIZYM (Biomerieux, UK)	
	Electrophoresis	
	Spectrophotometry	
	Mass spectrophotometry	
	Crystallography	
	ELISA	
Metabolite profiles	TLC	Metabolic profiles and active biomolecules
	HPLC	
	GCMS	
Antibiotic producing strains	As above:	Organisms with potential for antibiotic production
	Plus toxicity testing	
	Biological screens	
Product production	Fermentation	Bulk extract production
	Purification	Pure chemical library
	Chemical structure/characterization	Identified properties and uses
Biological control agents	Pathogenicity testing	BCAs as products for use in agriculture, horticulture, the environment
Biodeteriogens	Media and material based	Organisms to provide clean-up solutions
Utilizers, biodegraders, or bioremediators	Media based	Environmental biotechnology solutions
Tolerant, resistant, or sensitive strains	Media based	Assay strains or strains able to function in extreme environments
Chemical bioconversion/biotransformation	Media/fermentor based	Organisms to provide biotechnological solutions in industry
Fatty acids	MIDI	Enhanced identification database
Genetic characterization	RFLP	Genomic information to help in the "gene hunt"
	RAPDs	
	Sequencing	
	DGGE	
	Electrophoretic karyotyping	
	Microarray	

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4 CONCLUSIONS

Modern BRCs are more than just collections of living organisms; they are sources of knowledge, information, and expertise. The BRCs are adding value to the strains they hold through the application of characterization programs and by improvement of the methodologies by which they preserve their holdings. The services provided continue to expand but there is still much to do in relation to mycology, where the gap between what is known and the potential that is yet to be discovered and released is enormous. Despite the lack of qualified mycological systematists, the application of improved techniques associated with the integration of molecular techniques with traditional taxonomy is allowing progress to be made. However, the interest in mycology must be re-kindled to demonstrate the vast potential that fungi possess to provide the solutions to today's problems in agriculture, environment, public health, and world economy.

Sometimes, BRCs appear to be encouraged to work together to achieve common goals yet competition and commercialization seems necessary for their very existence. The *ad hoc* way in which collections access organisms has led to overlap in holdings and duplication of effort. Initiatives to co-ordinate collections nationally are well established but the huge task of making the species and information available on all organisms cannot be achieved by one country alone. The UK National Culture Collection (UKNCC) and the Belgian Co-ordinated Collections of Microorganisms have done a lot to develop joint policies and strategies. The European electronic catalog project "Common Access to Biological Information (CABRI)" has begun to develop this on an international scale. The regional, European Culture Collection Organisation (ECCO), and worldwide, World Federation for Culture Collections (WFCC) collection organizations have endeavored to foster collaboration. However, the key driving force to establishing strong international linkages and common strategies could be the OECD Biological Resource Centres Initiative. International agreement has been reached and recommendations made that should allow the formation of a Global Electronic Biological Resource Centre. However, it is not clear how this will work when competition between centers for the culture sales market is increasing. Many governments have removed support for national collections over the last 20 years, so the commercial imperative has been more important. The OECD Initiative has discussed specialization and indeed setting a focus for a BRC is essential as no one collection can hold representative strains of every species nor do they have the specialist expertise to describe, characterize, and handle them all. If collections did specialize then overlap would be reduced and collections would have their own niche in the market. However, some groups of organisms have stronger market potential than others so who would want to be responsible for those that are not currently *in vogue*.

There could be several financial models for the sustainability of these networked collections. Support could come from a mixture of sources, e.g., commercial sales of products and services, government support for national services provided, philanthropic sponsorship, and business partnerships. Certainly collections add value to their holdings and must continue to do so and share the benefits of the exploitation of properties and products discovered as culture sales alone is not a sustainable business. New technologies and changing problems put pressure on collections to adapt to the new demands upon them. Conservation will not pay for itself, perhaps BRCs as opposed to the traditional culture collection can characterize and utilize their holdings to better effect and still provide the key function of underpinning the life sciences providing the tools for biotechnology through the preservation of genomic integrity.

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Benefits and Risks of Genetically Modified Foods: An Overview

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1 INTRODUCTION

Since the move from hunter-gather to agriculturist from as long as 10,000 years ago, (Diamond 1998) human beings have been intervening in the heredity of plants, animals, and more recently microorganisms. Until the past decade this has involved selective breeding, including use of techniques such as controlled pollination, hybridization, and lately cloning. The genetic makeup of plants has thus undergone changing through breeding for several millennia (Halford and Shewry 2000). Random events such as genetic recombination and transposition of genes can occur spontaneously within plants. Chromosome rearrangements can be produced and mutations induced using chemical and irradiation treatments. Cross-species hybrids are more the norm than the exception in the botanical world (Royal Society of New Zealand 2000). Interspecies and even intergenera hybrids have been created using cell and tissue culture techniques such as embryo culture and *in vitro* fertilization (Scott and Conner 1999a,b).

The 1950s saw the discovery of the structure of DNA by Watson, Crick, and Wilson, and this has led to the ability to determine the genetic code of organisms. Isolation of individual genes was achieved in the 1970s. These advances paved the way for techniques that allow genetic coding to be altered in a manner not achievable through natural evolution or selective breeding.

Genetic modification (GM), also known as genetic engineering (GE), is the process by which an individual gene (or more commonly a set of a few genes) that controls particular characteristics is taken out of the DNA of one organism and inserted into the DNA of another organism. This allows for transfer of genes between species as well as within species (Burke 1998). The gene is isolated from a strand of DNA from a cell, changed, and then inserted into another cell of a plant, animal or microorganism.

Gene transfer can be achieved by several different methods, including the use of microorganisms, and by using a technique which shoots microscopic gold particles coated with genetic material into new cells (Halford and Shewry 2000). Microorganisms carrying novel genes may also be used to manufacture organic molecules, such as vitamins (Koizumi et al. 2000; Schulz and Beubler 1989) or enzymes, (Mala et al. 1998; Ross et al. 2000; Wesley 1981) for use in food processing.

GM differs from traditional selective breeding in three main ways: (a) In GM individual genes are extracted, may undergo some alternation, and then are transferred from one plant or animal into the DNA of another, (b) GM allows plants or animals to gain new inheritable qualities in a way that is much faster and sometimes much more novel than possible with selective breeding techniques, and (c) genes may be transferred between different species, genera or even kingdoms (between plants and animals) in a way not possible in nature (Royal Commission on Genetic Modification 2001).

The rapid development of GM science has promoted intense public and professional debate. On one hand, GM technology may offer huge benefits in food production, improving both the quality and quantity of foods produced and helping to alleviate poverty and starvation. On the other hand, it may be a dangerous genie we are letting out of the bottle, with unforeseen dangers and risks to the health and well-being of individuals, populations and our planet (Burke 1998; Editorial 1999; Holden 1999; Millstone et al. 1999; Mowat et al. 1999; Trewavas and Leaver 1999).

This chapter examines the role microorganisms play in GM food technology; attempts to identify the potential benefits and hazards of GM foods; and discusses issues regarding regulation of this technology.

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2 GM FOODS AND MICROORGANISMS

2.1 Use of Microorganisms in the Transfer of Genes

To transfer genes using a microorganism, DNA strands are extracted from a cell, proteins known as restriction enzymes are added to break the DNA at particular points to obtain an individual gene, and then this gene is placed inside a bacterial cell. The genes are incorporated into plasmids, small DNA molecules that are not chromosomal in nature, and which can move between cells, not the whole plasmid! carrying the gene with them. Bacteria carrying the added gene can then transfer this new gene to target plant or animal cells, whose DNA is recombined to incorporate the new gene. To identify cells with the desired gene, an accompanying 'marker' gene is necessary. Typically, these selective markers have been antibiotic-resistance genes rendering the transformed cells resistant to an antibiotic, which is toxic to cells not genetically modified (Halford and Shewry 2000). Thus, all the cells in the target tissue that are not genetically modified are then destroyed in the lab.

2.2 Use of Microorganisms in the Production of Ingredients

Microorganisms carrying novel genes may serve as factories for large-scale production of desired organic chemicals used in food production. In the metabolic engineering of microorganisms, genes may be mixed from several different organisms to produce hybrid structures. This engineered strain is then used *in vivo* to produce the desired metabolites. A growing field is genetically engineered synthesis, which involves a microorganism acquiring the genes with their recombination *in vitro* and subsequent production of the desired compounds without requiring a host organism (Roessner and Scott 1996).

There are an increasing number of possible uses of microbes in the production of ingredients for food through the use of specific enzymes and fermentation processes. Proteases are degradative enzymes that catalyze the hydrolysis of proteins into amino acids. They are found in all living organisms and have wide application within the food industry. They have been routinely used for centuries in processes such as cheese-making, baking, tenderizing of meat and production of soy products such as soy sauce. Protease extraction from plant and animal sources is time-consuming and expensive, hence the genetic manipulation of microorganisms for protease production has become an important alternative source (Mala et al. 1998). Proteases can be commercially produced using bacteria (especially *Bacillus* genus), fungi (particularly useful in cheese-making) and viruses (particularly for medical rather than food applications).

For example *Bacillus thermoprotolyticus* is used to synthesize aspartame, a widely used noncalorific artificial sweetener (Mala et al. 1998; Wesley 1981). The GM use of glucoamylase genes from *Saccharomyces diastaticus*, *Rhizopus oryzae*, or *Aspergillus awamori* has produced a brewers' yeast with an altered ability to degrade starch, resulting in a lower digestible carbohydrate content giving a reduced calorie beer for diabetics (Schulz and Beubler 1989).

The enzyme chymosin is also being produced by microorganisms. Chymosin cleaves to milk protein (casein) to allow curdling in cheese-making and traditionally it has been sourced from rennet extracted from calves' stomachs. Recombinant chymosin has been produced by isolating the chymosin gene and introducing it into a microorganism (for example, *Bacillus subtilus*, *Aspergilllus niger*, or *Mucer michei* (Mala et al. 1998) and then manufacturing large quantities of the enzyme through fermentation. Recombinant chymosin has become a widely used alternative to calf rennet. Cheese produced using recombinant chymosin is acceptable to some vegetarians who will not eat cheese made with rennet (Ross et al. 2000).

Microorganisms carrying specified genes can also be utilized to synthesize vitamins. For example, *Corynebacterium ammoniagenes* has been used to carry plasmid-containing riboflavin biosynthetic genes and this has resulted in a many-fold increase in the production of riboflavin (vitamin B2) (Koizumi et al. 2000). Genetically modified yeasts have been used to change the vitamin and alcohol content of beer (Schulz and Beubler 1989).

Genetic manipulation of microorganisms is also used to produce food flavorings. For example, in response to a world-wide decline in vanilla production, vanilla flavoring is being produced by genetically modified food fermentation organisms (Narbad and Walton 1998).

3 POTENTIAL ADVANTAGES OF GM FOODS

The GM foods may primarily benefit commercial interests but there are also possible direct gains to consumers. Specialized "nutraceutical" foods are being developed with a range of potential benefits for human health.

3.1 Improving the Processing and Marketing Qualities of Food

Some of the advantages of GM food primarily benefit food growers and manufacturers. The food industry might profit from GM technology by many ways. Improving the sustainability, efficiency, and cost-effectiveness in food production leads to improved productivity. Changes can be made to properties affecting storage to extend the shelf-life of a product. For example, a GM slow-ripening tomato has been extensively used in the manufacture of tomato paste (Tucker 1993). Introduction of a gene that neutralizes the pectin-degrading enzyme retards the softening process. This results

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in tomatoes that remain firm and sweet for seven to ten days longer than ordinary varieties (Rose 1992).

In developing countries beset with problems of over-population and poverty, the yield improvement rate of crop production is reaching a plateau while the rate of demand is increasing exponentially (McLaren 2000). Transgenetic crops may help provide a stable and sustained production of high quality food by increasing the yield, and improving the nutritional quality (Potrykus et al. 1995). The development of crops which require less water to thrive, or which can grow in saline-polluted soil is being explored (Glenn et al. 1999).

While these developments may be driven by the commercial interests of the food industry, consumers may also gain from increased yield of GM foods with a greater continuity of supply and possible lower prices (Halford and Shewry 2000; Jones 1999; Scott and Conner 1999a,b).

3.2 Producing Foods with Reduced Allergenicity and Lower Toxin Levels

Both selective breeding and GM techniques can result in random and unexpected genetic events. However, GM techniques introduce only one or a few genes into the crop, in comparison to conventional cross hybridization, which has the potential for multiple introduction of undesirable genes (Feldbaum 1999; Trewavas and Leaver 1999). Since it is known what protein a specific gene makes, the novel protein in transgenetic plants can be quantified, tested, and evaluated for its possible allergic or toxic qualities to an extent not possible with conventional breeding methods. Foods with reduced allergenicity therefore could be produced. Allergic reactions to foods such as cereals, nuts, milk, and eggs can be life-threatening in hyper-sensitive people. Transgenic varieties of grasses, birch, and oilseed rape have been created with reduced anaphylactic activity by susceptible individuals (Singh et al. 1999). These are used in allergy immunotherapy, where patients are given increasing amounts of disease-eliciting allergens in order to develop reduced allergen-specific responsiveness. It therefore may be possible to create edible cereals with similar reduced hyper-sensitivity in reactive people.

Proteases from *Aspergillus oryzae*, a *Rhizopus* species and a *Bacillus* species have been used to produce hypoallergenic casein, with an antigenicity of about 10,000 times lower than that of standard cow's casein, for possible use in many kinds of milk-containing foods and hypoallergenic baby formulae (Nakamura et al. 1991).

A hypoallergenic rice has been developed that has the same palatability and nutritional value as normal rice for people who suffer from rice allergy (Arai 1993).

3.3 Improving the Nutritional Value of Food

The GM technology may increase the nutritional value of food. Vitamin A and iron deficiency is prevalent in Third World countries, causing considerable morbidity, including blindness, and mortality. Rice is the staple food for much of the world's population. Standard milled indica rice is very low in Vitamin A. A transgenetic rice ("golden rice") is been developed with increased β-carotene (the precursor for vitamin A) and iron content, with the hope that it will provide a cheap accessible form of supplementation of these deficiencies (Ye et al. 2000).

3.4 Foods with Medicinal Properties

Foods are being developed by both conventional and GM means designed to enhance health-promoting effects of foods. This includes health maintenance and prevention; performance improvement; disease risk reduction; symptom self-treatment and disease intervention. Such products may be classified as functional foods, nutraceuticals, medicinal foods, and dietary supplements, although precise definitions of these terms have not been established and one may blend with another.

"Nutraceuticals" generally refers to products which enhance nutrition (for example, vitamin A-enriched rice). A functional food is one intended for consumption as part of a normal diet, but contains modifications that may contribute to disease prevention (such as adding plant sterols to margarine). Another example is a GM potato that has been developed with an increased starch and decreased water content that can halve the amount of oil absorbed during frying (Rose 1993).

Digestion of the lactose in milk requires the presence of the enzyme lactase, which is produced in the small intestine. Lactose intolerance is caused by a deficiency of this enzyme. Malabsorption of lactose is a common problem causing nausea, diarrhea and abdominal pain. Genetic modification research is currently developing the expression of the lactase enzyme in bovine mammary glands, which converts the lactose to glucose and galactose. The resultant milk has the appearance and qualities of standard milk but greatly reduced lactose levels (Whitelaw 1999).

Examples of foods with potential medicinal properties are profuse and the possibilities are expanding all the time. Although they may be shown not to fit the definition of "panacea" previously acclaimed, many traditional food remedies are being shown to have valid pharmaceutical actions. For example, research on green tea (from *Camilla sinesihas*) suggests that it may have antimicrobial, immunostimulatory, anticarcinogenic, antiinflammatory, and cardiovascular-protective capacities (Sato and Miyata 2000a–d). Green tea may have some antimicrobial capacity against various bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Vibrio cholerae*, *Escherichia coli*, *Streptococcus salivarius*, and *Streptococcus mutans*. Garlic (*Allium sativum*) has also been shown to have antimicrobial action against bacteria including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Salmonella typhimurium*. In addition, garlic has been shown to have antifungal properties (Sato and Miyata 2000a–d). The

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antimicrobial activity of garlic is attributed to allicin. Allicin has also been shown to have cholesterol-lowering effects. Honey may also have antimicrobial properties, although its antimicrobial ability differs depending on its floral source (Sato and Miyata 2000a–d). Studies indicate that ginseng has immunostimulatory and anticarcinogenic properties. It contains a compound called ginsenoside, which appears to be immunologically active (Sato and Miyata 2000a–d).

Genetic modification could potentially enhance the medicinal properties of these foods. For example, the level of allicin in garlic could be increased to improve its cholesterol-lowering capacity. The GM foods are also being developed with increased concentrations of components such as carotenoids, omega fatty acids, and flavonoids. There is growing evidence that these phytonutrients have a positive impact on human health (Kochian and Garvin 1999).

Dietary supplements are products that contain extracts, concentrates, or synthetic versions of food substances, such as vitamins, minerals, amino acids, herbs, or synthetic nutrients and are usually sold in controlled dose forms such as powders, capsules or tablets. Some of these may be produced using GM technology.

3.5 Reducing the Use of Chemicals in the Growing Process of Foods

The development of pest and disease-resistant crops results in the reduction in use of pesticides. The aim is reduced residue in the food as well as a reduction of chemical pollution of the environment (Potrykus et al. 1995). Glyphosate-tolerant crops allow the use of a single safe herbicide instead of the need for more toxic chemicals, and have led to reduction of total herbicide use by 50% in some cases (Halford and Shewry 2000).

3.6 Foods with Improved Taste

A GM soybean has been developed that is lipoxygenase-free. Soybeans lacking lipoxygenases have increased storage stability. Furthermore, lipoxygenase isozymes are responsible for the beany flavor and bitter taste of soybeans and this GM soybean has the potential in the manufacture of soy yoghurt, soy milks, ice-cream, and light-plain tofu products that are more palatable to consumers who do not like the traditional soy taste (Kitamura 1995).

4 POTENTIAL RISKS OF GM FOODS

Potential risks of GM foods may be to individual consumers, to human populations or to the environment.

4.1 Risk of Producing Allergenic Foods

There is no evidence that in general GM foods will be more or less allergenic than their corresponding conventional foods (Wal 1999). However, because GM involves the transfer of genes, which may code for proteins not normally present, it is possible to transfer allergenicity from the host to the new variety in specific situations, and hence bestow an allergen in a food to which an individual was not previously allergic.

In a number of cases where transgenic varieties were tested for allergenicity, no difference in allergen content was found between wild and transgenic strains (Lehrer and Reese 1997). Two such cases are a new variety of corn with altered amino acid composition which was tested on subjects known to be corn-reactive; and similarly a transgenic soybean with an altered fatty acid profile was tested on soy-allergic individuals.

One case where an allergen from a known allergenic food has been transferred into another food by GM involved the transfer of methionine-rich 2S albumin from brazil nuts into soybeans, to improve the nutritional quality of the latter, which are relatively deficient in methionine (Nordlee et al. 1996). Because Brazil nuts have a known allergenicity, a comprehensive testing regime was undergone. Subjects allergic to Brazil nuts were tested for allergy to the transgenic soybean and were found to test positive. Although the soybeans were intended for animal rather than human consumption, the company abandoned the project to ensure that the modified soy did not enter the human food chain (Jones 1999).

Of greater concern, however, was the development of StarLink™ corn in 1998 (Dorey 2000). The gene which encodes for an insecticidal Cry9C protein was inserted in this corn using GM *Bacillus thuringiensis* (Bt). Because there were some concerns that Cry9C protein may be allergenic, the corn was approved for animal feed only. However, in the year 2000 fragments of the Cr9C DNA were found in corn taco shells in the United States, which were subsequently recalled. Subsequent research indicated a possible risk that the Cry9C protein is a potential allergen (Anonymous 2001), although this has not been confirmed. In this instance, there was no risk to human health. Only the encoding gene, not the protein, was found in the taco shells. Moreover research indicates that the Cry9C protein is present in StarLink corn kernels at a level of 0.3% (and hence would be considerably $<0.3\%$ in the taco shells) whereas most allergenic proteins are present at levels of 1–40% (Dorey 2000). It is thought that the detected Cry9C genes resulted from physical contamination, although the possibility of cross-pollination in the field has not been eliminated. This case raises issues about the difficulty of restricting a GM food for animal use when there is a similar non-GM equivalent available for human consumption (Royal Commission on Genetic Modification 2001).

The GM researchers contend that when the gene source is from a known allergen, it is possible to determine whether the allergen content of the transgenic line is altered relative to the

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nontransgenic varieties using traditional forms of immunological testing. If allergens are transferred to a new variety, this can be established before the food is made available to unsuspecting consumers (Lehrer and Reese 1997).

Opponents to GM technology counter-argue that GM may create or unmask new immunoreactive structures. If the gene source is not a known allergen, traditional forms of immunological testing may not be applicable. To date there is no completely reliable and objective method (using either animal testing or chemical analysis) to evaluate or predict the possible allergenicity of a new protein (Wal 1999). Research is underway to determine whether food allergens share physicochemical properties that distinguish them from nonallergens. If this is so, these properties may be used as a tool to predict the inherent allergenicity of proteins newly introduced into the food supply by GM. One study has found that major allergens of plant-derived foods such as legumes (peanuts and soybean) are stable to digestion, in comparison to nonallergenic food proteins (for example, spinach) (Astwood et al. 1996). This means that digestible proteins are unlikely to be allergens.

4.2 Risk of Producing Toxic Food

While there are no established cases of food toxicity resulting from GM, there are two cases that have raised concerns. The first involves an epidemic of eosinophilia myalgia syndrome (EMS) in 1989 that resulted in 37 deaths and over 1500 nonfatal reported cases. These cases have been clearly linked to ingestion of a specific batch of the food supplement tryptophan manufactured by the Japanese company Showa Denko KK (Kilbourne et al. 1996; Slutsker et al. 1990). The tryptophan dimer L-tryptophan was identified as a contaminant and is believed to be the causative agent in EMS (Buss et al. 1996).

Tryptophan is produced by a fermentation process, which involves growing large amounts of bacteria and then extracting and purifying the amino acid. The company had made two changes in their manufacturing process prior to the outbreak. They had started to use a GM bacterium *Bacillus amyloliquefaciens* that had an enhanced production of tryptophan. They had also altered the extraction and purification process by reducing the amount of activated carbon used in filtration by 50% (Scott and Conner 1999a). Because Showa Denko reportedly destroyed their stocks of the GM bacterium strain once the EMS cases began to emerge, unfortunately it has not been possible to definitively establish whether the dimer L-tryptophan contaminant (and hence the EMS outbreak) specifically arose from use of the GM bacteria or from an *inadequate purification* process (Belongia et al. 1990). There has been heated scientific debate as to whether EMS epidemic was triggered by an impurity formed when the manufacturing conditions were modified, or was GM-related, (Henning et al. 1993; Mayeno and Gleich 1994; Scott and Conner 1999b) but because the GM strain was destroyed, the answer will remain unknown.

The second case involves GM potatoes being used in a research project. In 1998 a British scientist Arpad Pusztai prematurely announced through the media the unpublished results of a study describing intestinal changes in rats which had eaten these GM potatoes (Anonymous 1999). This led to considerable public concern about the dangers of GM foods. A review of the available Pusztai data conducted by the Royal Society concluded that there were methodological flaws in the research and misinterpretation of these results (Lachmann 1999). The potatoes were not being developed as a food source in this particular study that was examining whether lectins, which make some plants unpalatable to insects, could have a similar affect if introduced into other plants. This issue still remains controversial (Mowat et al. 1999).

4.3 Risks GM Foods May Pose to Human Populations

Concerns have been expressed that GM food may pose threats to human populations. For example, GM processes may produce foodstuffs that are selected for qualities that improve marketability but have reduced nutritional value. Of more wide-spread concern has been anxiety that GM processes may cause development of antibiotic resistance.

Sometimes in the GM process a "marker" gene is used that confers resistance to a certain antibiotic. This means that the antibiotic can be used to kill off all cells that have not undergone GM. The "marker" gene can later be removed from the GM food. It has been hypothesized that the use of antibiotic-resistant markers could pose a threat to the effectiveness of antibiotics in people and animals through its horizontal transfer to other organisms, including microbes which inhabit humans.

Generally the gene and the enzyme it produces that renders the antibiotic inactive are eliminated in subsequent processing (Advisory Committee on Novel Foods and Processes 1994). In addition, generally the antibiotics used are not those used to treat human infections. Concerns that this resistance could be transferred to human pathogens (British Medical Association 1999) has led to regulatory controls placed on further development of this product in Britain.

The issue of GM "markers" rendering human antibiotics ineffective has been extensively investigated by the World Health Organization and several European expert advisory panels. The general conclusion is that the impact on human health is effectively zero, although the issue still needs to be addressed on a case-by-case basis because it depends on the nature of each particular GM (Australia and New Zealand Food Authority 2000). However, in response to concerns, alternative marker systems are being developed to phase out the use of antibiotic markers (Advisory Committee on Novel Foods and Processes 1994).

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4.4 Risks GM Foods May Pose to the Environment

Concerns have been raised that the development of GM crops with resistance to herbicides may lead to this resistance being passed on to weeds though cross-pollination, resulting in herbicide-resistant "superweeds" (Nestle 1998).

One case that has undergone comprehensive testing is Monsanto's genetically modified soybeans that are able to resist the herbicidal action of Roundup. This food has been extensively produced in the United States and there appears to be no evidence to date that it is causing "superweed" development. There are reports however that GM oilseed rape plants have passed on their herbicide tolerance to neighboring weeds (Mikkelsen et al. 1996).

Other GM crops are bioengineered to contain the naturally-occurring insect toxin Bt. The Bt has long been used, particularly in the organic food industry, to protect agricultural and forestry crops from insect pests. Concern has been expressed that widespread use of the Bt gene might result in the selection of Bt-resistant insects. One case is reported of large-scale plantings of transgenic Bt cotton falling victim to cotton bollworms, one of three pests that the crops were supposed to kill, heightening fears that the insects will eventually develop resistance to the toxin (Kaiser 1996).

One long-term study of four different crops (potato, maize, oilseed rape, and sugar beet) grown in 12 different habitats and monitored over ten years did not find that the GM crops (with GM traits of resistance to herbicides or insect pests) were any more invasive or persistent than their conventional counterparts (Crawley et al. 2001).

5 ASSESSING BENEFIT/RISK RATIOS

5.1 Safety of GM Foods Compared with Food from Non-GM Varieties

Most conventional foods have not been tested. Our food crops have been developed using conventional breeding methods for centuries or even millennia, and hence assumed to be safe (Halford and Shewry 2000). However, plants have frequently evolved to contain toxins to protect them against predators, thus conventional foods contain toxins naturally, and can be poisonous to humans if not prepared properly. Examples include kidney beans, cassava, and Jamaican akee. Green potatoes contain poison, and dried beans require soaking and cooking in fresh water to remove the toxic lectins they contain. Despite their potential toxicity, these foods are staples for large sections of the world population.

Traditional breeding may carry risks to health. For example, a new variety of celery bred to be more disease-resistant caused skin rashes on a large percentage of grocery workers who handled the vegetable (Berkley et al. 1986). Foods can be contaminated by toxins produced by fungal activity before harvesting or during storage. Mycotoxins such as aflatoxin carry a carcinogenic risk (Campbell and Stoloff 1974). Ironically, organic foods that are not treated with fungicides are more at risk of containing these mycotoxins.

Genetic modification is a very precise process. The products of introduced genes are easily identifiable. The protein produced should be able to be purified, tested and monitored in the new food. In foods created by conventional breeding in contrast, the process is much more random and varied, making identification of changes in the composition of the food very difficult or impossible (Halford and Shewry 2000). Mutogenesis through chemical and irradiation treatments, the source of most modern hybrids, results in the recombination of large numbers of genes in unpredictable ways. By this argument, GM foods may be safer than foods derived from non-GM food techniques and should require no more testing than that applied to conventional foods.

5.2 Risks Involved with Foods Compared with Pharmaceuticals

Some contend that GM foods should undergo testing as stringent as that required for pharmaceuticals. When a new medical intervention is introduced, evidence is required about its efficacy (how effectively it produces the desired health effect) and its safety (the relative frequency and severity of possible adverse effects). With the development of new drugs, there are usually clear-cut desirable outcomes that can be measured. The health potential risk of a particular drug from adverse side effects is offset by the desired medical effects for a particular health problem. Ideally the benefit/risk ratio strongly favors the use of the drug in appropriate cases.

A particular medication is not usually designed for general human consumption, but only for the population of people in need of it for health reasons. Pharmaceutical products may be subject to a number of restrictions of use: to specific ages (for example, may be deemed not suitable for children or the elderly), to patients suffering from conditions the particular drug is promoted to assist; and defined upper limits for dosage and /or duration of use. The exact chemical composition of the drug is known, which may greatly facilitate its testing and monitoring.

In contrast, foods may contain hundreds of different compounds, with the exact composition unknown. Once introduced into the market, foods become available for consumption by the entire population, including the very young and the very old, with little or no control on how much or how often a particular food is consumed by some people.

However the boundary between food and medicine is becoming increasingly blurred, with the development of a number of products consumed for health and medicinal benefits (Murphy 1997). In general, the development, sale and monitoring of pharmaceuticals is much more stringently regulated than for foods. Dietary supplements are regulated under New Zealand statute as food not medicine. Other products such as functional foods do not have statutory

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regulation. However there is no clear distinction between what should be considered a food and what should be considered a drug. Many of the specific criteria for a drug (such as a given dose for a give age for a given condition) do not apply to functional foods, where there may be no control on who consumes the product, or on the amount or frequency with which it is consumed. Whereas GM foods are relatively stringently regulated, whole foods from traditional sources or new sources other than GM are not subjected to testing at all.

5.3 Benefit/Risk Ratio

Risk assessment involves estimation of both the probability and the consequence of an adverse event. Opponents to GM food promote the precautionary principle, which although variously defined, effectively states that where potential adverse effects are not fully understood, an activity should not proceed. However, no activity is risk-free. There is currently no evidence that GM foods present significant threat to consumer safety, although the potential exists for the creation of a GM food containing unexpected allergens or toxin. Any potential risk needs to be compared with known risks of everyday living. Food from any source may pose risks, both short-term (contain infective organisms, toxins, or allergens, cause choking) or long-term (for example high cholesterol diets may contribute to heart disease). Risk to the environment is a different and more complex issue and issues such as the potential development of "superweeds" need to be assessed on a case-by-case basis.

The benefit/risk ratio needs to take into consideration who is taking the risk and who is getting the benefit. Currently it is likely that GM food-producers and the multinational companies who buy their produce benefit more than individual consumers. New foods are likely to be more acceptable to consumers if they can identify direct benefits to themselves (food that is cheaper, tastes better, improves health) rather than gains by the food industry.

6 REGULATION

6.1 Moral and Ethical Issues

A high degree of public concern has been expressed towards GM technology, especially GM food. The GM foods are developed primarily for commercial gain, not for the direct benefit of consumers. Large corporations are seen to be motivated by their own financial interests and suspected of under-estimating or ignoring risks to human health to protect their own profits. Public mistrust of commercial industries has some validity. The tobacco industry is an example known to all.

The food industry can threaten human morbidity and mortality in many ways. Some risks are well known and regulations can prevent contamination and ensure food safety. However unforeseen risks also occur. In Britain a new variant form of the degenerative central nervous system disease Creutzfeldt-Jakob Disease (CJD) appears to be linked with consumption of beef infected with bovine spongiform encephalopathy (BSE), popularly known as mad cow disease. The BSE epidemic seems to have resulted from feeding the animals contaminated meat and bone meal. The official report was highly critical of the government agencies minimizing the risk to humans and being too slow in mounting a response (Royal Commission on Genetic Modification 2001).

Objections to GM technology include views that species should be considered sacrosanct and that combining the genetic material of two species which do not breed "in nature" is immoral, against God's will and/or potentially ecologically dangerous. This argument is countered to some degree by the proliferation and public acceptability of interspecies horticultural crop hybrids, e.g., wheat/corn that have been created over the past few centuries but may have more validity with the combination of much more dissimilar species.

The anti-GM food argument is often accompanied by emotive images such as a potato with frogs legs (Butler 1999) or a pumpkin with chicken legs (Kibbell 1999) designed to act on people's emotions rather than their intellects. People presented with these images may not appreciate that much of GM food technology involves production of an enzyme or other food component, or enhancement of an existing characteristic of a food, and not necessarily the amalgamation of two vastly different food groups. The role microorganisms plays in much GM technology is also poorly understood. These pictures do however raise important ethical issues of where one "draws the line." For some it may be acceptable to cross different plants that do not normally inter-breed, but not different animals or plants with animals; for others tampering with any species may violate their religious beliefs.

On the broader scale, genetic technology such as cloning and xenotransplantation (transplantation of tissues or organs from animals to humans for medical reasons) raises huge moral dilemmas regarding the nature of what it is to be "human" and the sanctity of human life, but beyond scope of this discussion.

6.2 Labeling

People may chose to avoid GM foods for ethical, cultural, or religious reasons. It can be argued that people have a right to know what they are consuming, and where possible should be able to chose whether or not they eat GM food. There is clearly public demand for labeling, whether or not it is scientifically justifiable, and the food industry needs to respond to the consumer's right to chose (Nestle 1998). Nestle argues that labeling of GM foods, premarket notification of about new GM products, and development of monitoring and testing regimes that restore public confidence would ultimately benefit the industry as well as the public.

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One option is labeling foods "GM-free" where producers can prove this is the case. Some consumers may be prepared to pay a premium for such a guarantee.

The New Zealand and Australian response has been an amendment of the Food Standards Code requiring mandatory premarket safety assessment and labeling (Australia and New Zealand Food Authority 2001). Labeling is required where novel DNA and/or protein is present in the final food; and where the food has altered characteristics. Foods containing up to 0.1% of GM material are exempt.

These measures do not satisfy consumer advocacy groups. For example, the New Zealand Green Party considers these regulations inadequate and is calling for all food products involving GM technology to be labeled, irrelevant of the presence of novel DNA/protein in the final product (The Green Party of Aotearoa/New Zealand 2000). On the other hand, some food producers find this regulation excessive and onerous, and claim that the process of tracking the source of all ingredients in some processed foods could result in significantly increased consumer costs.

6.3 Testing and Monitoring

There is need for rigorous and continuous testing, including the use of animal trials, to ensure that innovative food products are not hazardous. Close monitoring of the chemical purity of biotechnology-derived products should occur, particularly following any significant changes to the manufacturing process (Mayeno and Gleich 1994). Testing and monitoring need to consider the broader issues relating to the effects on the environment with rigorous safeguards to prevent uncontrolled release of GM organisms that may have detrimental effects on the ecosystem.

The US Food and Drug Administration (FDA) decided in 1999 that GM crops should receive the same consideration for health risks as any other new crop plant (Center 1992). This recommendation is not accepted by opponents to GM foods who argue that existing systems of checks and regulations are not adequate, and believe that GM foods need additional testing compared to conventionally produced novel foods (Millstone et al. 1999; Trewavas and Leaver 1999).

The risks involved in consuming foods (both GM and non-GM produced), functional foods, dietary supplements, and medicines are similar and vary only with the concentration of a component within the product and the quantity and frequency of its consumption. Therefore, an argument can be made for a common regulation of products for human consumption, with the same standards of testing, monitoring and ongoing surveillance applying to all. The assessment should be undertaken on a case-by-case basis, taking into account individual variables and circumstances. This is the position reached by the New Zealand Royal Commission on Genetic Modification (Royal Commission on Genetic Modification 2001) and is in line with the recommendations of the comprehensive report compiled in Britain (Nuffield Council on Bioethics 1999) that advocated local solutions for local people.

Clearly the regulatory process must be transparent. The preferable model would appear to follow that used in pharmaceutical regulation, where the testing and monitoring is done by the company. The role of the government is to set the criteria and protocols and ensure that the regulations are strictly adhered to.

7 CONCLUSIONS

The GM technology is the progression of millennia-long processes of human intervention in the heredity of plants, animals, and more recently microorganisms. It allows the transfer of genes between different species, genera, or even kingdoms in a way not possible in nature.

Microorganisms may be used to transfer individual genes to target plant or animal cells. Microorganisms carrying novel genes may also serve as factories for large-scale production of desired organic chemicals used in cx food production.

Potential advantages of GM foods include improving both the quality and quantity of foods produced and helping to alleviate poverty and starvation. Development of pest and disease-resistant crops may reduce pesticide use. Drought or temperature-resistant crops may allow food production on land currently not utilized. Increased yield of GM foods may give consumers greater continuity of supply and possible lower prices. The GM foods might have improved storage qualities or improved flavor, reduced potential toxicity or hypo-allogenicity. The GM foods could have increased nutritional value or enhanced health-promoting effects.

Potential risks to consumers include creating an allergen in a food to which an individual was not previously allergic or introduction of a toxin. Potential threats to human populations include production of food stuffs with improved marketability but reduced nutritional value, or the development of antibiotic resistance through the use of "marker" genes. The GM crops may threaten the environment if they have resistance to herbicides, which is passed on to weeds though cross-pollination, resulting in herbicide-resistant "superweeds."

Most conventional foods have not been tested and many contain natural toxins requiring processing before consumption, yet these are staples for large sections of the world population. Foods can be contaminated by mycotoxins before harvesting or during storage. Food from any source may pose both short-term and long-term risks. The precise nature of GM technology means the products of introduced genes are identifiable and hence more easily purified, tested, and monitored for in the new food.

Development of pharmaceuticals requires evidence about its efficacy and safety before release, with the benefit/risk ratio strongly favoring the use of the drug in appropriate cases. The boundary between food and medicine is becoming increasingly blurred, with the development of a number of products consumed for health and medicinal benefits. Currently, regulation is much more stringent for the

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pharmaceutical than the food industry. However with the development of functional foods and dietary supplements there is no clear distinction between what is food and what is drug.

Risk assessment involves estimating both the probability and the consequence of an event. While the precautionary principle might be evoked, no activity is risk-free. To date, there is no evidence that GM foods present a threat to consumer safety, although potential risk to the environment is more uncertain. Any potential risk needs to be compared with known risks of everyday living with consideration of who is taking the risk and who is getting the benefit. New foods are likely to be more acceptable to consumers if they can identify direct benefits to themselves rather than gains by the food industry.

People may chose to avoid GM foods for ethical, cultural or religious reasons. At the least, labeling appropriate foods "GM-free" is desirable. There is need for rigorous and continuous testing, including the use of animal trials, to ensure innovative food products are not hazardous. The risks involved in consuming foods (both GM and non-GM produced), functional foods, dietary supplements, and medicines are similar and vary only with the concentration of a component within the product and the quantity and frequency of consumption. There is a case for common regulation of products for human consumption, with the same standards of testing, monitoring and ongoing surveillance applying to all. The assessment should be undertaken on a case-by-case basis, taking into account individual variables and circumstances. This regulatory process must be transparent.

The goals to improve the quantity, quality and safety of the food supply are laudable, but it is also recognized that the primary motivation of the bio-food industry is financial gain. While the consumption of GM foods may not be inherently less safe than food stuffs developed through traditional breeding techniques, public distrust runs high.

Like so many scientific developments, the GM of food has both potential benefits and potential dangers. Comprehensive public and professional debate is desirable. However, it is important that this discussion is informed by science, and that claims of both benefits and risks are evidence-based. Objectivity is required to ensure that the process is driven neither by the vested interest of the bio-technical multi-national companies on the one hand, nor ill-informed public fears on the other.

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Transgenic Mycoherbicides: Needs and Safety Considerations

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1 INTRODUCTION

The biocontrol of weeds was discussed by Boyetchko (2004), at great length in a comprehensive book (TeBeest 1991), and in excellent recent reviews by Auld and Morin (1995); Weston (1999). This chapter is an updated and highly condensed version of a chapter entitled "Molecular biology in weed biocontrol" (Gressel 2002), by permission of the publisher. Hypervirulence for the purpose of this chapter is defined as obtaining a higher level of virulence than found in the most virulent native strain of a mycoherbicide.

We must face up to the fact that almost all proposed biocontrol agents specific for major weeds in arable row crop agriculture have not lived up to expectations. Most of the successes described in the previous chapter have been in the control of alien weeds by introducing the pathogen that kept it in balance in its center of origin ("classical biocontrol"). In contrast, most of the important weeds of major row crops have become globally distributed, quite evolved from their cohorts at the centers of origin, and cannot be controlled by the classical methods. Pathogens have been isolated that prey on many of these major global weeds and can provide a modicum of control when huge levels of inoculum are applied. Usually $> 10^4$ spores per cm^2 are thus "inundatively" applied, which is more than four orders of magnitude than required for 100% efficiency of the initial application. Thus, most proposed organisms have not been sufficiently cost-effective, despite efforts in efficient production of inocula and formulating the inocula (Boyette et al. 1991; Quimby et al. 1999) and in designing application technologies. Biological control should seem to be an easier technology to perfect than herbicides; herbicides are used at relatively high efficiency. Few herbicides could be made to work at 10 times lower rate let alone a ten thousand fold lower rate. The difference in orders of magnitude between practice and theory should be the target for improvement of weed biocontrol agents.

A host-specific hypervirulent pathogen that controls a major row-crop weed to the extent that farmers require (i.e., similar to control achieved with a chemical herbicide) might have gone extinct soon after evolution, as would the target weed; dead weeds and dead mycoherbicides cannot reproduce. Considering the lack of success attained with potential inundative mycoherbicides, despite years of research (albeit at low budget), it has been suggested that a solution could come from genetically engineering hypervirulence genes into weed-specific pathogens, where the pathogen is kept from becoming extinct by preservation of inoculum in the laboratory (Gressel 2002; Kistler 1991).

1.1 Ecological Needs/Justification for Biotechnologically Upgrading Mycoherbicides

It is only by conferring some type of hypervirulence that biocontrol agents will have the ability to augment or compete with herbicides in row cropping situations. The ecological balance achieved with classical agents in an extensive pasture or forestry situation cannot suffice in intensive agriculture. Basically, a homeostasis is achieved. Despite fluctuations (Arditi and Berryman 1991), (which in themselves would be unacceptable to the intensive farmer), the homeostasis cannot be broken with the classical agents that have co-evolved for such a long period with their hosts (Dennill and Hokkanen 1990; Frank 2000). Indeed, it is the long-term co-evolutionary war between plants and their natural enemies that is thought to be the source of evolutionary diversity among plants (Rausher 2001).

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Engineering genes for hypervirulence into a classical agent may tip the balance away from the homeostasis, but such an agent may "put itself out of a job," and re-infestation will be needed when the weeds make a come-back. Thus, the relationship will no longer be classical, with a one-time infection, but closer to inundative.

2 NEEDS FOR ENGINEERING TO GREATER VIRULENCE FOR CONTROLLING WEEDS

Laboratory control of early stages of weed growth may not translate into an effective mycoherbicide. Weed populations in the laboratory are typically synchronous. Weed populations in the field are typically asynchronous with germination in a number of flushes. The farmer wants herbicides (and thus a mycoherbicide) to control a number of flushes, preferably until the crop canopy closes, precluding competition.

The use mycoherbicides is an orphan science, with little study of weed-pathogen interactions. Conversely, much research focused on understanding the molecular biology of infection of crops (Dangl and Jones 2001), and how to overcome these infections, while we need to know how to successfully cause epidemics. It is very important to understand the natural infection process, and there is much to learn from it. Phytopathologists have been studying the defenses in so-called compatible responses, where the fungus typically infects the crop. Those engaged in biocontrol of weeds have begun to learn that the terms compatible and incompatible are misnomers. Infection is not qualitative, it is quantitative with thresholds and then dose dependency. The molecular defense responses in compatible systems have been a barrier to greater success of biocontrol agents. As each barrier is removed, virulence is enhanced and less inoculum is needed. Thus, the more barriers removed, the greater the enhancement of virulence, often in a synergistic manner.

By default, we may learn much from molecular studies of fungal virulence to crop plants and animals. Many upregulations of enzymes are being recorded during pathogen attack. As the genes are known, the expression of the genes can be transgenically suppressed, and the suppression correlated with a lack of virulence. In this manner, it has been shown that the glyoxylate cycle and phospholipase are each required for *Candida* and *Mycobacterium tuberculosis* virulence in mammals (Ghannoum 2000; Lorenz and Fink 2001). Thus, the utility of molecular tools such as gene disruption for finding virulence genes has been demonstrated and genes are being pinpointed (Kwon 1998). For use in enhancing virulence of mycoherbicides it is necessary to demonstrate that: (a) overexpression will enhance virulence. This is not a foregone conclusion. For example, the lack of cAMP has been correlated with a loss of virulence (Kronstad 1997) but it is doubted that overexpression of this signal transducer will enhance virulence; it is more like an on/off switch; (b) that the genes will work in heterologous systems; and eventually (c) that weeds will not quickly evolve mechanisms to overcome the enhanced virulence. The latter is not a moot point when we realize that strain resistance to pathogens is often correlated with resistance to virulence enhancing toxins, (e.g., Pedras and Biesenthal 2000), which is analogous to well-documented evolution of herbicide resistance (Gressel 2002).

2.1 Weed Defenses Against Biocontrol Agents: Problems to Overcome

The weeds attacked by mycoherbicides initiate various defenses to suppress the spread of invading pathogens including: (a) PR (pathogenicity-related) proteins (chitinases and glucanases as well as other stress-related proteins) can be induced by pathogen attack. The chitinases and glucanases degrade fungal cell walls, preventing mycoherbicide penetration and growth in the tissue; (b) constitutive, or pathogen induced-phytoalexins, nonprotein, secondary metabolites of various chemistries that poison the fungus; (c) physical barriers composed of polymers such as suberin, lignin, callose, or their mixtures are induced. Often papillae of callose rapidly form, blocking the advancing fungal hyphae. Such papillae can contain small amounts of lignin. Traces of lignification can severely suppress degradation of carbohydrate polymers such as callose and cellulose by fungal enzymes (Gressel et al. 1983).

Different species initiate different defenses, and the levels of induced and pre-existing defenses can vary during weed growth and development. The outcome of this is that different quantities of inocula are needed at different stages of growth, a major complication to using biological control agents. Similar age dependencies often exist with some chemical herbicides, and herbicide chemists have strived to develop chemicals with less age dependency, a goal that must be paralleled with mycoherbicides.

There can be a strong genetic load to a pathogen from having a gene or genes introduced. Such a load might reduce growth rate (on rich media, or heterotrophically in the environment) but should be an advantage when attacking a weed. This genetic load could be deemed as positive, not just because it kills the weed. It is positive because from an ecological point of view (as well as the commercial point of view, which is immaterial to this discussion) it is better to have the transgenic organism dissipate from the environment when the weed is not present. This sounds like planned obsolescence, but it is really planned biosafety.

3 LESSONS FROM THE TRANSGENIC BIOCONTROL OF INSECTS AND DISEASES

The paucity of reports on transgenic biocontrol of weeds suggests that it may be worthwhile to take a cue from biocontrol of insect pests and diseases. A wide variety of

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transgenic viruses, bacteria, and fungi have been successful in controlling insects and diseases (Vurro et al. 2001a). Microorganisms have been doing this to each other throughout evolutionary history using, e.g., streptomycin (from Actinomycetes), penicillin (from mycelial fungi), and killer toxins (yeast). Many of the genes controlling their production are inherited on plasmids that are passed around among organisms.

Organisms can be engineered to higher levels of virulence without recombinant technologies; a strain of *Trichoderma* has been developed that effectively controls various plant pathogens; it is a heterokaryon fusion product between two other strains (Harman and Donzelli 2001). Similarly, mutant mycoherbicidal fungi that overproduce and secrete valine have been selected using amino acid analog antimetabolites such as norvaline or penicillamine. The resistant strains elicit the same symptomology as herbicides inhibiting acetolactate synthase (Sands and Pilgeram 2001).

The ideal mycoherbicide envisaged will contain a group of transgenes that will synergistically enhance; (a) mycoparasitism (cutinolytic and cellulolytic genes); (b) antibiosis (yeast killer toxin genes); (c) enhanced competition for nutrients or space (stronger siderophores); and (d) inactivation of the plant pathogens defensive enzymes (proteases). Some of these genes have already been engineered into biocontrol agents of pathogens (e.g., genes encoding chitinases) (Harman and Donzelli 2001), and into fungi that parasitize insect pests (St. Leger and Screen 2001).

Two approaches have been taken to improve the action of viruses on insects. Different nucleocapsid nucleopolyhedroviruses were tested for control of *Helicoverpa* and *Spodoptera* in cotton, but were too slow acting. Their efficacy was improved by using recombinant virus with the ecdysteroid UDP-glucosyltransferase (*egt*) gene deleted (Flipsen et al. 1995). The speed of killing was synergistically further improved by inserting the insect-specific toxin gene *AaIT* in the *egt* locus (Chen et al. 2000).

The lessons to be learnt from the transgenic biocontrol of insects and fungi are the same as being learnt with chemical herbicides: (a) Formulation or engineering for stability is important. BT toxin in its native *Bacillus thuriengensis* is UV-unstable. Engineering the toxin gene into a melanized, UV impermeable spore forming organism such as *Pseudomonas*, stabilized it. Conversely, perhaps the Bt itself could have been engineered to be melanized; (b) Transgene products that initiate cascades are preferable. Engineering a cuticle degrading protease into a hemolymph-attacking fungus activates a prophenoloxidase system (St. Leger et al. 1996); (c) Containment is important. The prophenoloxidase system described in (b) removes the substrate needed for fungal sporulation, limiting spread (St. Leger et al. 1996); (d) Selectivity is important. Host specificity should not be altered by transgenically enhancing virulence. Thus, potency is elevated while selectivity is retained; and (e) Synergies are important for decreasing inputs as discussed above. Nature typically uses multiple mechanisms both to synergistically protect organisms or to synergistically prey on other organisms. Plants transformed to produce both BT and chitinase (Regev et al. 1996) or with pea trypsin inhibitor (Fan et al. 1999) kill later larval instars with less transgenic protein than can be achieved by high levels of BT alone.

Resistance to pathogens has been engineered into crops by inserting the chitinase that appears in the *Trichoderma* strain used for biocontrol (Lorito et al. 1998; Lorito et al. 2001). Three way synergies have been shown in antipathogenic mycofungicides. cDNAs encoding three barley proteins; a class-II chitinase, a class-II beta-1,3-glucanase and a Type-I ribosome-inactivating protein were expressed in tobacco plants. There was enhanced synergistic protection against *Rhizoctonia solani* infection compared to isogenic lines expressing a single barley transgene to a similar level (Jach et al. 1995).

4 BIOTECHNOLOGICALLY UPGRADING BIOCONTROL AGENTS

Organisms can potentially be modified to increase pathogenicity by transformation with genes for virulence from other species, by increasing the endogenous expression of genes, or by transfer from other organisms by protoplast fusion (Gressel 2002; Harman and Stasz 1991; Harman and Donzelli 2001; Kistler 1991). Increasing virulence, especially by gene transfer, requires extreme care due to environmental impact, i.e., the possibility of increasing the host range to include crops. Thus engineering "failsafe devices" into the pathogens (See "Section Prevention of Persistence and Spread of Transgenic Mycoherbicides") may be a necessity.

4.1 The Possible Needs to Upgrade the Crops

One consideration for enhanced biocontrol is to utilize nonspecific, general mycoherbicides. For example, the use of an asporogenic mutant, amino acid auxotrophic *Sclerotinia* was proposed as a general mycoherbicide, that could not spread or reproduce in the field beyond the target (Sands and Miller 1993). It was never developed beyond the experimental stage, probably languishing because it was not virulent enough. Enhancing its virulence might turn it into the glyphosate of a decade ago; an organism capable of total weed control—but with a limited market. The markets of glyphosate and other herbicides was enhanced manifold by engineering crops to be resistant to the herbicides (Gressel 2002). A corollary could be considered for mycoherbicides, transgenically generate a crop specifically resistant to a broad spectrum mycoherbicide. *Sclerotinia* is a heavy excreter of oxalate, which is an important (Zhou and Boland 1999), but not sole determinant of pathogenicity (Callahan and Rowe 1991). A gene for overexpression of oxalate decarboxylase has been engineered into crops, conferring resistance to *Sclerotinia* (Kesarwani et al. 2000). Plants engineered to overproduce oxalate oxidase were also resistant to fungal attack (Zaghmout et al. 1997).

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Another case to consider: the broad spectrum mycotoxin deoxynivalenol targets L3 protein in 60S ribosomes. Plants transformed with a modified RPl3 gene were resistant to the mycotoxin (Harris and Gleddie 2001).

5 GENES THAT MAY ENHANCE WEED BIOCONTROL

Biocontrol agents could also be engineered to convey genes that convert proherbicides to herbicides. This would then allow the mycoherbicide to be applied together with the pro-herbicides. Such a gain of function mutation has been engineered into the tobacco etch virus (TEV), encoding a $P450_{su1}$ that activates a sulfonylurea proherbicide (Whitham et al. 1999). The same gene could also be put into any other biocontrol agent. Systemic and complete infection may not be needed, if a systemic herbicide is released by the activated gene product. This would have distinct advantages with weeds with underground propagules such as *Cyperus* that regrow after foliar killing. As the biocontrol agent is specific to the weed, it would not cause the conversion of proherbicide to herbicide in crops.

Many genes are becoming available that might be appropriate for enhancing the activities of mycoherbicides. Some single genes that may be appropriate are summarized in Table 1. At one time, it was thought that it would be problematic to transform organisms to produce complicated molecules such as nonprotein toxins because of the large number of genes involved in the pathways to produce such secondary metabolites. Eukaryotic organisms do not have polycistronic gene sequences as in bacteria. Thus, the concept of complex molecule biosynthesis seemed daunting because of the need to hunt genes scattered over the genome. Indeed genes for primary metabolism are scattered over the genome in fungi unlike their polycistronic nature in bacteria. This seems not the case for toxin-biosynthesis genes; they are clustered (Kimura et al. 2001; Walton 2000). One of the concepts supporting the possibility of horizontal gene transfer in fungi is based on the same clustered synteny of secondary metabolite biosynthesis in unrelated organisms (Kimura et al. 2001; Walton 2000). Thus, with luck one may be able to isolate and transform such clustered complex pathways into biocontrol agents.

Genes encoding characterized biochemical functions will be summarized below, but there are also known virulence genes isolated that have no known function. For example, double stranded (ds) RNA similar to and related to cryptic viruses has been correlated with fungal virulence in *Nectria*. Virulence is lost in "cured" strains from which the dsRNA disappeared and was restored through heterokaryon anastomosis (Ahn and Lee 2001). If/how such elements can be moved among organisms, and whether they will induce hypervirulence is yet an open question.

Hypervirulence genes that can possibly be used "off the shelf" to try to enhance biocontrol agents may be classed as "soft," i.e., those genes whose products dissolve the weed host or detoxify its defense mechanisms, or that produce compounds which already occur human diet, and appear on the official "GRAS" (generally regarded as safe) lists, and are lightly regulated. There is a spectrum of possibilities from these to very "hard" genes producing strong toxins. The regulatory agencies should look with care more than once at organisms with "hard" genes, but with an open mind. Many of these genes may produce compounds that are toxic to mammals, but only at particular life stages during weed growth, so there would be little danger. If a biocontrol agent containing such a gene does not enter the food chain and it is no less dangerous to the applicator than conventional herbicides, there exists a good reason to attempt the regulatory hurdles.

Not all agents are at the soft and hard extremes; lytic enzymes that eat holes in the weed may be very similar or identical to the same GRAS enzymes used in processing of

Table 1 Simple genes that may be appropriate for enhancing biocontrol activity of weeds by microorganisms

Gene	Property	References
Soft genes		
tomatinase/*pda1*	Degrade phytoalexins	Roldan et al. (1999); Ruan and Straney (1996)
iaaH	Auxin biosynthesis	Robinson et al. (1998)
iaaM	Auxin biosynthesis	Comai and Kosuge (1982)
Xalicae	Inhibit callose synthase and other enzymes	Robinson et al. (1998)
Cbp1	Binds calcium, depriving host	Sebghati et al. (2000)
Intermediate[a]		
Branch chain amino acid	Secrete branch chain amino acids, effect similar to ALS inhibitors	Sands and Pilgeram (2001)
P450 sul	Converts pro-herbicide to herbicide	Whitham et al. (1999)
Hard		
Nep1	Protein causes leaf necrosis	Bailey et al. (2000); Jennings et al. (2000)
Yeast killer genes	Will they kill plants?	Schmitt and Schernikau (1997)

[a] See also Table 2.1 in Gressel (2002) for organisms containing genes that are known to have plant-specific toxins.

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food and beverages. Also genes producing already commercial and safe herbicides might be in the middle.

5.1 Soft Genes

5.1.1 Transgenically Overcoming Host Defenses

Much is known about pathogen genes encoding enzymes that degrade crop phytoalexins or constitutive fungal toxins, enhancing virulence. For example a *Fusarium* tomatinase degrades α-tomatine of tomato (Roldan et al. 1999), and *pda1* gene of *Nectria* encodes a cytochrome P450 that degrades pisatin in peas (Ruan and Straney 1996). Overexpression of such genes could enhance virulence of mycoherbicides. Only rarely have the phytoalexins of weeds been determined (Sharon et al. 1992b) allowing knowledgeable choice of chemicals to suppress of phytoalexin production (Sharon et al. 1992a).

Often such phytoalexin-degrading genes are activated by the phytoalexins themselves, and the isolated promoters are specific to the phytoalexins (Khan and Straney 1999). Sands and Pilgeram (2001) have suggested that such promoters be used to empower other hypervirulence genes, ensuring host specificity of the hypervirulent biocontrol agent (unless a related crop makes the same phytoalexin).

5.1.2 Transgenically Causing Hormone Imbalance in Weeds

At present there is scant evidence to know what genes will work, but there is circumstantial evidence to say that some should work. For example, it was presumed that organisms overproducing the auxin IAA should enhance virulence. This is based on the findings that: (a) Exogenously-applied IAA can lead to cellulase-catalyzed cleavage of hemicellulose, resulting in wall loosening (Taguchi et al. 1999) and membrane leakage, thereby stimulating the loss of water and nutrients (Brandl and Lindow 1998); (b) Synthetic auxins such as the herbicide 2,4-D bind auxin receptors; (c) Many microorganisms associated with plants as symbionts or parasites directly alter the auxin content of the host; the auxin imbalance benefits the microorganisms (Gaudin et al. 1994). Fungal-infected tissue can contain ten times more auxin than the level found in healthy apices (Gruen 1959); (d) Pathogen strain virulence directly correlates with the levels of IAA, suggesting that IAA has a positive function in the infection process. Plants preinfected with a pathogen caused auxin production, which then overcame the hypersensitive defense response of plants subsequently infected by an incompatible pathogen (Robinette and Matthysse 1990). IAA deficient mutant strains are not pathogenic, but virulence was restored when these mutants were transformed with genes for IAA synthesis (Comai and Kosuge 1982); and (e) Chitinase produced by plant tissues as a defense against pathogens was blocked by auxin, enhancing the virulence of the pathogens (Shinshi et al. 1987).

Thus it was worth testing the effects of overproduction of any and all plant hormones in mycoherbicidal organisms based on the assumption that plants have evolved to have optimal levels of hormones. Any vast hormone oversupply should at least cause imbalances that will facilitate pathogen establishment, growth, and development.

In two cases devoted to biocontrol of a weed, there was a modicum of success. *Fusarium* spp. transformed with two genes of the indole-3-acetamide pathway leading from tryptophan to IAA produced significantly more IAA than the wild type and were more effective in suppressing the number and size of *Orobanche* shoots than the wild type (Cohen et al. 2002). The same genes greatly enhanced the activity of a *Colletotrichum coccodes* attacking *Abutilon*, but only when augmented with tryptophan (Amsellem and Gressel, unpublished results 2002). This demonstrates that having an enzyme may not be sufficient, if substrate for the enzymes is lacking. Similarly, when the gene *ipt* or other genes inducing cytokinin biosynthesis were introduced into plants causing an overproduction, the plants were dwarfed with small leaves (Hlinkova et al. 1998). Hormone levels were modified by inserting rice homeobox OSH1 into tobacco, again severely disrupting growth processes with minimal amounts of add hormone (Kusaba et al. 1998). Would the same happen with these genes in fungi, and would such a disruption of hormonal balance enhance mycoherbicide action?

5.1.3 Dissolving Host Defenses

As described in Section 3, "Lessons from the Transgenic Biocontrol of Insects and Diseases," cell wall degradases have been used to enhance virulence against insects and pathogens. The importance of phospholipases in fungal pathogenesis in mammalian systems has been extensively reviewed (Ghannoum 2000), and extracellular lipases are part of virulence of some fungi attacking plants (Eddine et al. 2001) and perhaps their up regulation could enhance virulence.

A rapid increase in callose biosynthesis is typically seen in compatible responses to *Colletotrichum* infection. Lignin-like material later becomes embedded in the callose, sterically preventing its degradation by glucanases, and thus inhibiting the progression of infective fungi (Kauss 1992). Callose synthase can also be inhibited rather specifically by deoxyglucose, enhancing the virulence of a pathogen (Stanghellini et al. 1993). Alas this is only effective when applied through cut surfaces, the antimetabolite does not penetrate plant cuticles.

The enzyme callose synthase has calcium as an obligate cofactor, and the activity of this enzyme *in vitro* can be blocked by chelators (Kauss 1992). Fungi have very low requirements but high affinity for calcium. The requirement for calcium is so low that *Colletotrichum coccodes* could be grown for generations on putatively calcium-free media containing EGTA, a very strong calcium specific chelator. It was hypothesized that infecting a weed with a compatible *Colletotrichum* mycoherbicide together with a calcium

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chelator would enhance infectivity (Gressel et al. 2002). As most calcium chelators are too hydrophilic to penetrate plant cuticles, EGTA derivatives were synthesized with hydrophobic tails and applied with *Colletotrichum* to *Abutilon*. Some of these compounds as well as calcium-complexing oxalic acid, doubled the number of infection sites (Gressel et al. 2002). Concomitantly, microscopic analysis showed that far less callose was present after such treatments. A similar enhancement of mycoherbicide virulence was found by augmenting the formulation with oxalate (Briere et al. 2000; Watson and Ahn 2001). Thus, calcium deprivation increased infectivity while decreasing callose content. This correlation fits the hypothesis but does not prove it, as calcium deprivation can have many effects in plants. Still, there is considerable evidence that oxalate is naturally used by fungi as part of their pathogenesis process (Dutton and Evans 1996). Oxalate can be synthesized in fungi: (a) by cleavage of oxaloacetate from Kreb's cycle (TCA cycle) by the enzyme oxaloacetase (Müller 1986); (b) oxidation of glyoxylate by glyoxylate NADP-1 oxyreductase (glyoxylate dehydrogenase) (Akamatsu 1993); and (c) by oxidation of ascorbate analogs; erythroascorbate and its galactoside by ascorbate oxidase (Loewus 1999). These genes could be considered for transgenically enhancing virulence. They are ubiquitous in fungi yet not all seem to be producing oxalate, so there is either a question of regulation and/or secretion.

A database search suggests that the genes responsible for oxalate production in fungi have not been isolated. The enzyme glyoxylate dehydrogenase has been completely purified, and is dauntingly large (331,000 D) (Tokimatsu et al. 1998). The gene will probably not be large, as the enzyme is composed of six identical homopolymers.

It is valid to ask whether increasing hypervirulence through oxalate production by the biocontrol agent should be transgenic, based on two arguments: (a) Perhaps it is metabolically and economically "cheaper" to provide commercial oxalate in the formulation than produce it from metabolites in the biocontrol agent. Such production can be at the expense of growth; and (b) providing oxalate in the formulation confers transient hypervirulence. The spores later formed do not have greater virulence. Using exogenous oxalate can act as a failsafe mechanism and preclude the necessity to register a transgenic organism. These arguments may be valid for any gene product, and should be part of the considerations for any over production. Oxalate is especially inexpensive to provide, compared to enzymes and protein toxins.

There is always the possibility that a target weed will evolve resistance to oxalate overproducers, or to added oxalate. The oxalate catabolic enzyme oxalate decarboxylase from *Collybio velatipes* (a basidiomycete) has significant sequence homology with germin-like proteins from *Arabidopsis*, tomato, and rice (Kesarwani et al. 2000). Oxalate can also be oxidized in plants by oxalate oxidase. Engineering poplars to overproduce oxalate oxidase rendered the plants resistant to *Septoria* (Liang et al. 2001). Conversely, overexpression of a calcium binding protein (Sebghati et al. 2000) might have the same effect as oxalate, and will be harder to overcome.

5.2 Hard Genes Encoding Toxins

Fermenter produced fungal toxins have been proposed for use as stand alone "biocontrol" agents. The term biocontrol is best limited to the use of a living organism, and the possibility of using separately produced fungal derived phytotoxins alone or as adjuvants to enhance virulence of mycoherbicides will not be discussed, and the reader is referred to (Abbas et al. 2001; Bailey et al. 2000b; Duke et al. 2001; Vurro 2001; Vurro et al. 2001b) among others. This chapter will deal only with toxins produced by the mycoherbicide itself that enhances its virulence.

Complex fungal toxins pre-dated herbicides, and were the lead compounds for herbicide discovery (see Gressel 2002—Table 2.6). The genes encoding their production and that of novel toxins (Kimura et al. 2001; Vurro 2001) can be considered for enhancing mycoherbicide virulence, if indeed the genes are clustered, which seems to be the case in many instances (Kimura et al. 2001; Walton 2000). Another, easier approach is to use peptide toxins that can be produced in a single step from a single gene. Indeed, virulence was increased 9 fold without losing host specificity by introducing *nep1*, a gene encoding a phytotoxic protein, to an *Abutilon theophrasti*–specific, weakly mycoherbicidal strain of *Colletotrichum coccodes*. The parent strain was at best infective on juvenile cotyledons of this intransigent weed. The transgenic strain was lethal through the three-leaf stage, providing the time window of control of this asynchronously germinating weed (Amsellem et al. 2002).

A major problem in the engineering of mycoherbicides for toxin production may well be in obtaining expression. Some of the most potent toxin producing fungi, produce their toxins only when cultured on media, but not when they are pathogenic. We found that culture filtrates of strains of *Fusarium oxysporum* were toxic to tomato, yet the fungi grow on the tomato rhizoplane, specifically attacking *Orobanche* spp. (broomrapes) before and after they parasitize the tomato (Cohen et al. 2002). Similarly, *Myrothecium verrucaria* produces milligrams of tricothecenes per gram medium in culture. Highly sensitive technologies could not find traces of these toxins when the pathogen infected kudzu (*Pueraria montana*) (Abbas et al. 2001). If the toxins are necessary for fungal virulence, they must be made in a very localized area for a very short time; when needed for establishment, and toxin production should be under a contact inducible promoter. Additionally, transgenes encoding toxin degradation under control of a senescence activated promoter could be introduced into mycoherbicides to clear dead weeds of toxins, as an additional failsafe mechanism (as discussed later).

Even before such specific efficient promoters are found, it seems that it may be possible to use native promoters, but in distantly related species. Various *Fusarium* and other fungal

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species produce phytotoxic NEP1 proteins. The *nep1* gene was reintroduced with high expression promoters and made high levels of NEP1 on artificial media; so much so that NEP1 was considered for use as a "natural herbicide" (Bailey et al. 2000a) or as a separately produced additive to enhance virulence of another mycoherbicidal organism (Bailey et al. 2000b). The over expressing transformants did not have enhanced virulence when introduced into *Fusarium* used to infect a weed, only making toxin on artificial media (Bailey et al. 2002). The same construct enhanced virulence ten fold (i.e., a tenth as much inoculum was needed compared to wild type) when introduced into a *Colletotrichum coccodes* specifically pathogenic on *Abutilon theophrasti* (Amsellem et al. 2002). The *nep1 Colletotrichum* rapidly killed through the three leaf stage, where at best, the wild type slowly killed plants with cotyledons or at most in the true leaf stage. A single example hardly proves that heterologous overexpression and hypervirulence is more likely than homologous overexpression, but it does point to a direction worthy of consideration.

5.2.1 Are Toxin Producing Mycoherbicides Inherently Dangerous?

There can be cases where toxin production may be thought to restrict the use of a mycoherbicide, i.e., when the toxin has high mammalian toxicity and may find its way into the food chain, as in the case of trichothecene production in a gorse-controlling agent (Morin et al. 2000). Pathogenic strains were found that did not produce these toxins. As described previously, tricothecene-producing strains did not produce toxin when infecting the target weed, only when they were in artificial culture. Such results suggest that toxicology must be based on real world field data, and not artificial media. If an organism produces toxins only when in artificial culture and the agricultural produce is toxin free, the organism is only hazardous to the producer of the mycoherbicide. This poses a conundrum. Some contend that such strains should not be used. Others counter by reminding that a very nontoxic herbicides such as glufosinate is produced from very dangerous organophosphates, and the only danger is to the production workers. Safe production facilities can be constructed to produce inoculum, just as safe factories produce glufosinate.

5.3 Should We Look at Up-Regulated Genes in Pathogenesis?

Well before the terms genomics and proteomics were coined, researchers were trying to ascertain what genes and gene products were "turned on" in plants during pathogenesis. If they were not activated in nonpathogenic strains, especially in mutants, they were considered to be pathogenesis-related. The question to be asked in our context is; will there be hypervirulence if the expression of genes that encode enzymes that counteract PR proteins is transgenically turned up? And then, what other genes must be active? And then, is there a controlling element that can be introduced into an organism that will up-regulate the endogenous genes, without a necessity to add more genes? Thus, one could up-regulate the genes for a branched amino acid biosynthesis, without the need of a mutagen. With advanced knowledge, such enhancement could be performed by site-directed chimeraplasty (Zhu et al. 2000) without leaving transgenic traces.

Another open question is whether it will be simpler to transform a biocontrol agent than to up-regulate a silenced gene. For example, in a recent effort to enhance the toxicity of the *Fusarium oxysporum* that attacks *Orobanche* spp. by transforming it with the *nep1* toxin producing gene, it was found by PCR that the putative biocontrol organism has an (apparently) silenced *nep1* gene in it (Z Amsellem and J Gressel, unpublished). Will this be the case with the genes controlling oxalate production and other genes that could potentially be effective in overcoming weed defenses?

6 RISKS FROM TRANSGENIC BIOCONTROL AGENTS

While little thought and effort have been previously focused on risk analysis of transformed mycoherbicides, there has been considerable discussion on the use of "transgenic arthropods as biocontrol agents of other arthropods" (arthropod natural enemies). Hoy (2000) summarizes at length the potential risk issues discussed in a workshop devoted to that subject that should be dealt with before releasing transgenic arthropods. The issues brought up are valid for all types of biocontrol agents and should be a part of any risk analysis.

6.1 Constraints on Using Molecularly-Enhanced Biocontrol Fungi

In nature, genes can move among fungal species by whatever means. Thus, there is good reason to assess the risks of such movement, as there are many good reasons to desire that organisms will not transfer hypervirulent transgenes from a biocontrol agent to a pathogen of a crop. If there is such transfer, then there is reason to desire that the transferred transgene will not become established in a nontarget population.

Many fungi with the potential to act as biocontrol agents exist as narrow host-range-specific pathogens that are related to pathogens of beneficial species. These *formae specialis* or pathovars have often been shown by DNA comparisons to have evolutionarily diverged a very long time ago from related pathogens. A case in point is the ability to show that most divergences in the imperfect *Fusarium oxysporum* with its thousands of *formae specialis* and even more vegetative compatibility groups occurred eons ago (Baayen et al. 2000). In contrast to this ancient evolution, there is evidence that

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parasexual recombination between closely related species and supposedly incompatible strains of the same species is more prevalent when environmental stresses are exerted (Julian et al. 1999; Molnar et al. 1990). Indeed, incompatibility between strains and even between species can be overcome by the mutation of a single gene (Leslie and Zeller 1997). This has occurred in both imperfect strains of *Fusarium* and *Rhizoctonia* (Basidiomycetes) species that include major crop pathogens as well as host-specific pathogens of weeds, some of which could be used as biocontrol agents if their virulence were to be enhanced. Some imperfect *Fusarium* spp. can be physiologically stimulated to sexuality (with the sexual forms called *Gibberella* spp.) and a gene for hypervirulence could then be moved sexually among strains that do not parasexually recombine by heterokaryon formation (Plattner et al. 1996). Interspecific protoplast fusion with the appearance of stable hybrids is known, e.g., between an insect-killing *Beauveria* sp. and a toxigenic species. Some of the somatic hybrids formed are hypervirulent (Viaud et al. 1998).

6.2 Is Horizontal Gene Transfer a Risk?

The possibility of horizontal gene transfer (asexual exchange among different species or incompatible strains) has been suggested as the cause for many anecdotal appearances of improbable genes or gene sequences in various fungi. The "implied" cases and the supporting evidence have recently been reviewed by Rosewich et al. (1999); Rosewich and Kistler (2000). They endeavored to exclude cases in which parasexual heterokaryon formation and protoplast fusion might be the cause, but as seen above, mutations and stress can alter sexuality and compatibility. For this reason it was recently proposed to refer to this "gray" area between vertical (sexual or parasexual) and horizontal exchange between closely related strains and organisms as "diagonal" gene transfer (Gressel 2000). An example of what might be true horizontal gene transfer is the circumstantial EST (expressed sequence tag) evidence for the appearance of a bacterial chymotrypsin in a fungus (Screen 2000). Chymotrypsins were previously unknown in fungi, and sequence analysis showed

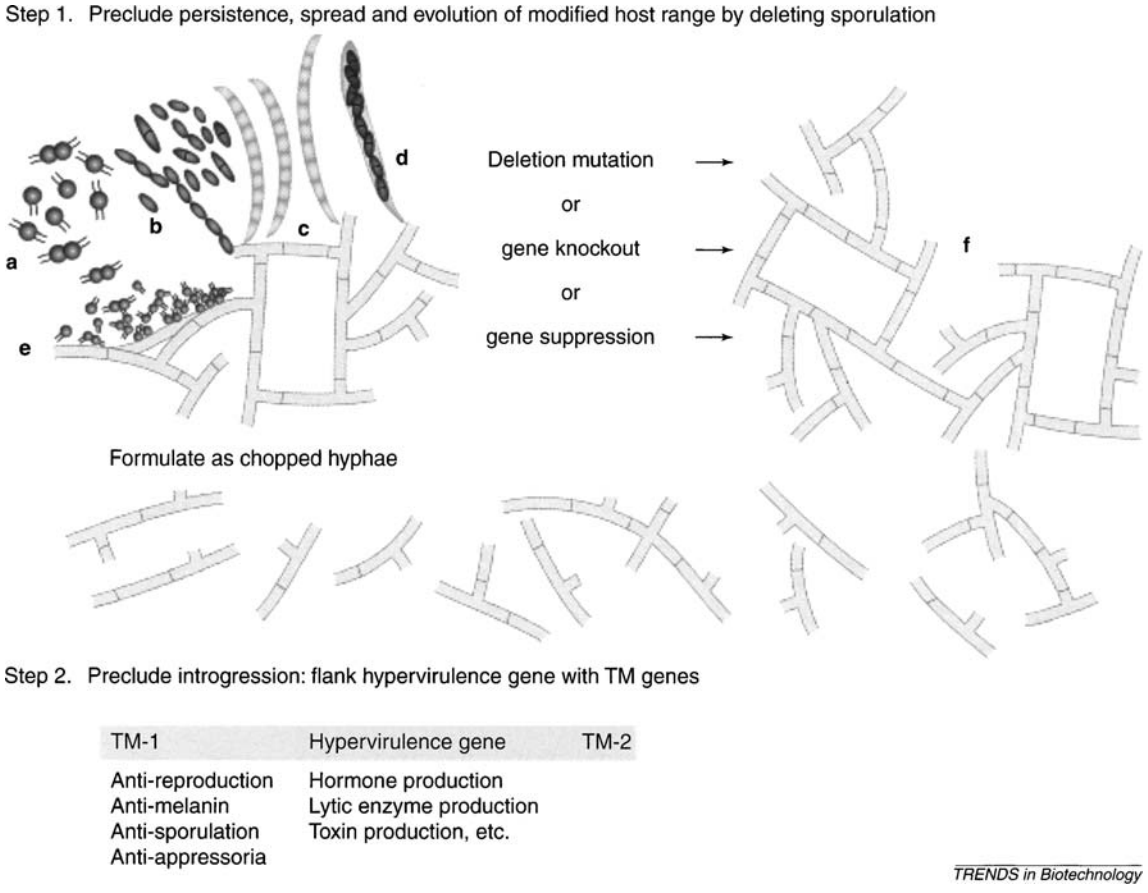

Figure 1 Dual failsafes to prevent (Step 1) spread of biocontrol agents, and (Step 2) their introgression into other organisms. a. chlamydospores; b. microconidia; c. macroconidia; d. ascus with ascospores; e. sclerotia; f. asporogenic mycelia (Source: From Gressel (2001) by permission.). Copyright 2001 Elsevier Science.

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that the intron-free gene of the fungus and that of a soil bacterium are related (Screen 2000). Rosewich and Kistler, after exhaustively discussing all the extant reports, conclude that horizontal gene transfer in fungi has "not been proven beyond reasonable doubt" (Rosewich and Kistler 2000), but diagonal gene transfer is a clear possibility, especially when organisms are under stress.

6.3 Potentially Acute Constraints

Three potential acute biological constraints have limited the interest in developing transgenic mycoherbicides: (a) The possibility that the agent would persist in the environment and spread, affecting nontarget hosts that were not assayed when checking host range; (b) they could mutate to a change in host range. Such evolutionary change in host range is potentially more hazardous in a hypervirulent organism than in the native pathogen; and (c) the mycoherbicide might sexually or asexually introgress and recombine with a related pathogenic species that attacks desirable species, and confer it with hypervirulence. Fungi can sexually mate or asexually conjugate or form heterokaryons and thus transfer genetic material to closely related species by "diagonal" gene transfer.

7 PREVENTION OF PERSISTENCE AND SPREAD OF TRANSGENIC MYCOHERBICIDES

Various methods can be considered to prevent persistence and spread. For example, transgenic gene disruption can be used to limit the ability of a mycoherbicide to exist in nature, but be used as a biocontrol agent. For example, *Psy1* disruptants of a *Trichoderma* could still be used as biocontrol agents against damping off agents, but they lack siderophores (encoded by this gene) that allow them to compete in natural, low iron environments (Wilhite et al. 2001). The gene could be knocked out in the transgenic mycoherbicide to prevent persistence and used in antisense or suppressive overexpression in a construct with hypervirulence genes to debilitate any organism that introgresses the hypervirulence construct, as described later.

A scheme to obviate the spread of native or host-range mutated agents, and an additional scheme to mitigate introgression are described (Figure 1). The general concepts described may be broadly applicable to biocontrol agents against other pest types. The specific examples presented are more limited to mycoherbicides. Fungi typically spend parts of their life cycles in dormant, resting structures that are resistant to heat, cold, desiccation, or other environmental tribulations. These same resting propagules (spores, conidia, sclerotia, pycnidia, ascospores, etc.) are a major form of dispersal, whether by wind, water, or animal movement. The suppression of spore formation whether by mutation (Sands and Miller 1993) or transgenically in hypervirulent biocontrol agents can prevent both persistence and spread (Figure 1). Nonsporulating mutants are not hard to isolate; it is probably best in such a case to use a physical mutagen (gamma or neutron radiation) that causes a loss of gene fragments. Point mutations caused by ultraviolet light or most chemical mutagens can revert, whereas deletions cannot. Experiments using mycoherbicidal levels of *Sclerotinia* highlighted the necessity to use nonsclerotia forming mutants; the half life of sclerotia was always more than 6 months, and it would take four years for the densities to decay to those found in pristine soils (Bourdot et al. 2001).

It was recently shown for two fungal species that chopped mycelia could be dried, stored for over a year and rehydrated (Amsellem et al. 1999). The rehydrated mycelia were more virulent than spores of the same species, because the mycelia establish more quickly in the pest (Amsellem et al. 1999). This procedure has an added asset insofar as it is usually far more efficient to produce mycelia in liquid culture than spores in liquid or on solid media.

The spread of mycoherbicidal agents can also be prevented by rendering them transgenically asporogenic (Figure 1a). This could be performed by antisense type strategies or preferably by gene targeting (Shiotani and Tsuge 1995) and knockout. Many pathogenic species seem to require melanized spores or structures for pathogenicity (Butler et al. 2001; Perfect et al. 1999). The germinating spores often develop melanized appressoria that attach tightly to the host, forming an infection peg that penetrates the host. Occasionally, the same species can attack both via melanized appressoria, as well as by mycelial penetration through stomates in the leaves (Latunde-Dada et al. 1999). Thus, if appressoria are not needed in the target weed for biocontrol, losing the ability to make appressoria would preclude some host range changes.

Mycelia themselves are not always pathogenic. Where only spores are pathogenic, the spread of a transgenic hypervirulent biocontrol agent could be prevented by a more complex strategy akin to the 'terminator' strategy (Crouch 1998; Oliver et al. 1998). Transgenes that could potentially suppress sporulation could be engineered into the biocontrol organism under the control of a chemically-inducible promoter. Sporulation genes in antisense configuration or in high overexpression so as to cause co-suppression would suppress the sporulation. Spores or mycelia to be used as inoculum could then be treated with the chemical inducer, before application to the target pest. The chemical inducer could be contained in the micropellet used for application (Amsellem et al. 1999), or the chemical inducer could be an endogenous, specific compound in the pest host. Thus, the biocontrol agent could be contained to the single, purposely-infested weed population. The use of transgenic sporulation suppression is less appealing than the use of physical deletion mutations because of the possibility of transgene silencing, allowing the organism to revert back to wild type.

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Table 2 Genes that might act as transgenetic mitigators™ if introgressed into nontarget pathogens in gene dependent orientation and regulation

Target process	(Presumed) mode of gene action	References
Potential TM gene		
Reproduction		
hetC	Heterokaryon incompatibility	Saupe et al. (1996)
sfaD[a]	Repressing G protein β subunit	Rosen et al. (1999)
stuA	Transcription factor	Wu and Miller (1997)
Appressorium formation[b]		
mpg1	Hydrophobic surface recognition protein	Talbot et al. (1996)
mac1[c]	Adenylate cyclase	Choi and Dean (1997)
pmk1	MAP kinase	Xu and Hamer (1996)
Spore stalk formation		
fluG	Unknown diffusible factor	Lee and Adams (1996)
brl A	Contains Zn finger motifs	Yamada (1999)
chsA/chsE	Chitin formation	Culp et al. (2000)
chk1[d]	Mitogen activated protein kinase	Lev et al. (1999)
Viable spore formation[b]		
abaA	Regulates phialide to spore transition	Sewall et al. (1990)
magC	G protein α subunit-decreases conidiation	Liu and Dean (1997)
cmp1	Spore surface protein	Puyesky et al. (1999)
acr1[d]	Prevents regulation of *mpg1* for spore maturation	Lau et al. (1998)
chsA/chsD	Chitin synthesis	Motoyama et al. (1997)
Spore germination		
cmk1[d]	MAP kinase (regulator gene)	Takano et al. (2000)
ctg1	G protein α subunit required for germination	Truesdell et al. (2000)
Melanin formation		
alb1	Polyketide synthase	Fujii et al. (1999); Motoyama et al. (1997); Takano et al. (2000); Tsai et al. (1999); Wu and Miller (1997)
arp1	Scytalone dehydratase	Kubo et al. (1996); Langfelder et al. (1998); Takano et al. (2000)
arp2	Hydroxynaphthalene reductase	Takano et al. (2000)
Mineral nutrition		
psy1	Iron siderophore production	Wilhite et al. (2001)

[a] Must be used in the constitutive sense form to suppress reproduction.
[b] No spores are produced when there are no spore stalks.
[c] Also reduces sporulation. Additionally, there are other spore-specific genes that are expressed, with yet unclassified phenotypes that may prove to be vital for spore function. These include *SpoC1-c1c* (Stephens et al. 1999).
[d] Also prevents appressorium formation.
(Source: Modified from Gressel (2001) by permission of Elsevier).

7.1 Obviating Recombination Between Mycoherbicidal Agents and Crop Pathogens

The previous strategies can be used to prevent persistence and spread, but would not preclude introgression with organisms from the same or related species (after sexual conjugation or heterokaryon formation). Such conjugation might provide genes that support spore formation, resulting in hypervirulent, persistent, and spreading pathogens. Thus, means are needed to mitigate the possibility that recombined, introgressed, hypervirulent organisms could become "superbugs" attacking nontargeted species.

A concept of using analogous tandem constructs was recently proposed as a failsafe to mitigate introgression of transgenes from crops to weeds (Gressel 1999) and has been modified to deal with biocontrol agents (Gressel 2001a). It was proposed that the important hypervirulence gene be flanked with transgenic mitigator (TM) genes that are positive or neutral to the biocontrol agent in the form to be used, but would be detrimental to any recombinant (Figure 1b). In the simplest form, as an example, the primary hypervirulence gene(s) could be flanked by one or two of the TM genes listed in Table 2, if the TM genes do not adversely affect virulence on the target weed. These genes, in the antisense or

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co-suppressive form would affect one of the processes leading to the ability to recombine, to form viable spores, or to make efficient infection structures. Some of these genes may have other deleterious effects that may render them inappropriate for this purpose. Clearly there are many known as well as yet to be discovered genes that would be appropriate additions to this list.

An antisense gene suppressing sporulation (Table 2) should prevent sporulation in a heterokaryon or other recombinant organism. The genes that control melanin biosynthesis and/or conidiation might only be applicable for biocontrol agents that do not need spores or melanin for pathogenicity. Spores without melanin do not have the viability in, or resistance to harsh environments. Thus, if some spores do form, they would be without vigor. Interestingly, related genes can have different functions. Of the three genes described coding for Gα protein subunits, deletion of *magC* had no effect on mycelial growth or appressorium formation, unlike *magA* or *magB* (Liu and Dean 1997; Truesdell et al. 2000).

8 RISK ANALYSIS AND LIMITATIONS OF THESE FAILSAFES

Risk analysis must be separately performed for each transgenic biocontrol agent considering two types of issues: (a) the limitations on the failsafe mechanisms that can be used; and (b) the biology of the pathogen and its relatedness to other pathogens.

A safety aspect that must be clearly ascertained early in development of asporogenic mutants or by antisensing spore formation, is that all types of sporulation are suppressed. For example, only light-induced conidiation is precluded in some asporogenic fungal mutants, but not starvation-induced sporulation (Horwitz et al. 1985). Some organisms make more than one type of spore; many *Fusarium* species can produce micro and macro conidia as well as chlamydospores. Each is produced under different environmental conditions. It would be interesting to ascertain whether each of the genes that control spore stalk development (Table 2) can (when antisensed) suppress all types of spore forms. The *stuA* transcription factor does control both sexual and asexual reproduction in *Aspergillus nidulans*, so genes are known that suppress all spore types (Wu and Miller 1997). It will be easier to load more or simpler failsafe mechanisms into organisms that do not require appressoria for penetration. This includes mycoherbicides that attack through stomates or other inter or intra cellular penetration.

More complex methods such as the modified "terminator" technology will also have to be considered for organisms that use melanized appressoria, or where hyphae are not typically pathogenic. Not all organisms (e.g., *Alternaria alternata*) utilize melanization of appressoria as part of infection (Kawamura et al. 1997). One can consider using a spore-specific promoter for antimelanin genes where hyphae form appressoria-requiring melanin. Thus, failsafe mechanisms will perforce be more complex with the melanized appressoria-utilizing *Colletotrichum* species (Perfect et al. 1999). Still, some *Colletotrichum* spp. can attack plants by stomatal penetration (Latunde-Dada et al. 1999). Many regulatory genes that are activated during sporulation are known (Adams et al. 1998; Marshall and Timberlake 1991). Such genes could be used to activate melanin suppressing or other antisporogenesis genes to render them more spore specific.

8.1 Risk Considerations Based on Pathogen Biology

Background knowledge about the possibility of a pathogen mutating its host specificity and its ability or inability to sexually or asexually conjugate "diagonally" with related organisms will govern the required number and level of failsafe mechanisms. Thus, one must consider the possibility of mutation causing pathogenicity to a broader host spectrum of a host-specific biocontrol agent, e.g., a specific pathovar of *Fusarium oxysporum*. There are no documented cases of a member of this species mutating its host range. The species is sub-divided into hundreds of known *formae specialis*, each with its own host specificity. There must have been evolution to different hosts, even if not documented cases, suggesting that more caution is needed here than for (example) the *Fusarium arthrosporioides* that is known to be pathogenic on *Orobanche* species (Amsellem et al. 2001). This *Fusarium* does not have known *formae specialis* (pathovars) on known crop species where it was isolated. Still, the possible existence of alternate crop hosts must be considered.

Some species easily conjugate with close relatives forming heterokaryotic mycelia with mixed nuclei (e.g., *Trichoderma*), and the mycelia have mixed properties that can enhance pathogenicity and host range (Harman and Donzelli 2001). Problems might ensue where spores are multinucleate or where there is recombination among nuclear chromosomes. Further generations will carry the heterokaryotic complemented properties. In a multigenerational experiment with hundreds of millions of (uninucleate) spores, there was no recombination among complementing nuclei that allowed a heterokaryon of two different *Trichoderma* auxotrophs to live on minimal media. No spore formed on these heterokaryons that could exist on this minimal medium (E. Galun, unpublished results). This demonstrates that even closely-related or con-specific organisms have impenetrable barriers to prevent recombination with "alien" genomes in nature, even when the traits could be beneficial or even vital for existence. Imperfect (asexual) fungi have less capacity to transfer traits than perfect (sexual) fungi. Still, it is impossible to "prove" that an imperfect fungus does not have a rare sexual form that appears only in highly special conditions.

Scientists and regulators interested in risk assessment would be advised to set up a decision tree mechanism that will

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allow an organized, less biased mechanism to formally answer a series of questions that will assist in categorizing risk. Such decision trees have been constructed for evaluating the analogous situation of introgression of transgenic traits from crops to related weeds (Gressel and Rotteveel 2000). It is imperative that containment (Kahn and Mathur 1999) be considered when working with transgenic hypervirulent organisms that can spread, until they are transgenically mitigated or otherwise deemed safe for release into the environment.

9 MARKING BIOCONTROL AGENTS

There are a variety of needs for devising simpler recognition methods for mycoherbicides and other organisms marketed in commerce; whether they are conventionally selected, mutant, or transgenic bacteria, fungi, plants or animals. The needs include: (a) *The need for protection for patented or other IP lines*, where IP takes on either designation: "Intellectual Property" or "Identity Preserved." It is often hard to prove that a line has been "miss-appropriated"; (b) *The need to trace biocontrol and other inoculation systems in the environment*. If the use of mycoherbicides increases, there will be a need for tracing irrespective of whether the mycoherbicide is indigenous or transgenic. Many of the mycoherbicides are closely related to known pathogens and proof (against grower liability suits) will continually be needed that it did change its host range and attack valuable species. There are complicated DNA fingerprinting techniques to accurately ascertain causality (e.g., Hintz et al. 2001; Inglis et al. 2001), but they cannot be used to answer the general question: "I suspect a mycoherbicide—which is it?" and; (c) *Labeling* Regulatory authorities and various consumer groups are demanding labeling of transgenic commodities. They spend vast sums typically probing for commonly used promoters or selectable marker genes and not for the trait genes. Even when transgenics are discovered with such "kits," there is no information as to source.

Thus, there is a need for common recognition sequences for detecting transgenic or other organisms released into the environment, in a single test that will clear or implicate particular organism. The simplest detection system for differentiating a large number of products is the "bar code" system. A simple genetic analogy has been proposed (Gressel 2001). A set of two universal "nonsense" (noncoding) nucleotide sequences is designed that can be detected by a set of universal PCR primers to recognize all "Biobarcodes™". The universal primers are long enough that a few mutational changes in the initial universal sequence will still allow it to be recognized by a PCR primer. The Biobarcode DNA can be co-transformed or spliced with the gene of choice. In other cases, an excisable selectable marker will be needed, so that just the Biobarcode remains after transformation. The PCR amplified Biobarcode can be automatically sequenced and compared to the Biobarcode database to ascertain the source of the organism. Should there be a possibility of introgression of the Biobarcode from the initial organism into another strain or species, RFLP or AFLP can be used to elucidate the target organism.

10 WEEDS WILL EVOLVE RESISTANCE TO TRANSGENIC-MYCOHERBICIDES

Weeds have been very successful in evolving partial resistance to the pathogens and arthropods attacking them, in a long-term evolutionary sense. Weeds are weeds because they are capable of replacing themselves in agroecosystems, despite generations of human intervention. In a thoughtful analysis "when is biological control evolutionary stable (or is it?)" Holt and Hochberg (1997) cogently ask why there is a lack of reports on the evolution of resistance to biocontrol agents. The mundane reasons for lack of resistance may reside in the fact that they have not been used long enough and to a large enough extent, or just poor reporting. Their discussion scrutinizes the long-term questions relating to "classical" agents, but can be extended to transgenic hypervirulent agents. In many respects the factors impinging on the evolution of resistance are analogous to those discussed (Gressel 2002) vis à vis the evolution of herbicide resistance. Their analysis (Holt and Hochberg 1997) is long and complex, and is discussed in the context of mycoherbicides in Gressel (2002).

In the final analysis, the strong selection pressure of transgenic hypervirulent organisms has the potential of selecting for resistant weed populations. The choice of genes and the synergistic use of multigenes can temper this evolutionary process, and crop/conventional/biological herbicide rotation should further delay such evolutionary processes, retaining transgenic hypervirulent mycoherbicides in the farmers' arsenal for a long time.

11 CONCLUSIONS

Mycoherbicides will only be widely used in most row crop situations if virulence is transgenically enhanced to overcome the evolutionary barriers that limit achieving adequate weed control. Enough genes are already available and the proof of concept has been demonstrated. Concepts for transgenically limiting off target spread and mitigating effects of gene flow, as well as universally marking hypervirulent mycoherbicides have been delineated.

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Recent Developments in Biotechnology and Intellectual Property, Access to Genetic Resources, and Benefit-Sharing

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1 INTRODUCTION

The field of biotechnology has seen rapid growth in recent years. As a result of recent scientific discoveries, genetic manipulation techniques have been developed that introduce the possibility of tailoring living organisms, and of transferring genetic material from one organism to another to create modified organisms that have certain "desirable" features; for instance, the modification of certain species of tomatoes and strawberries using a gene from a cold-water fish to protect the plants from frost. Such developments, in particular in the fields of medicine, food energy and the protection of the environment, have profound implications for society, and the debate surrounding the bio-ethics of this work will undoubtedly create headline news for many years to come.

Nonetheless, biotechnology, in particular fungal biotechnology, is one of the oldest known technologies. For instance, mycelial fungi and yeast have traditionally played a very important role in brewing and baking. Both are dependant on the conversion of sugar into alcohol and carbon dioxide by yeasts. Early processes were dependent upon contamination by "wild" yeasts; today, pure strains tend to be employed, and over 1 million tons of baker's yeast (*Saccharomyces cerevisiae*) are produced worldwide every year. Cheese production is also dependent upon the growth of moulds to develop the characteristic flavors of certain popular cheeses, such as Camembert, Roquefort, and Brie. Fungi are also well known as a source of antibiotics and citric acid. With the introduction of technologies, which enable fungal organisms to be genetically engineered, the practical applications of fungi have increased dramatically. Current developments in the field of fungal biotechnology include the use of fungi in new production processes that are considered less polluting than traditional chemical processes, such as the use of some species of white rot fungi to degrade toxic wastes, the use of fungi as biocontrol agents to kill insects (mycoinsecticides), and weeds (mycoherbicides), and the development of techniques that may enable textile dyes to be produced by fermentation.

This increase in the practical applications of biotechnology has been matched, in the last decade, by a transformation in international, regional and national laws, and policies relating to access to and the utilization of genetic resources. For instance, the 1983 International Undertaking (IU), which was negotiated under the auspices of the Food and Agriculture Organization (FAO) of the United Nations, and was the first comprehensive international agreement to address plant genetic resources for food and agriculture, has recently been revised to reflect the rapidly changing status of genetic resources. The International Treaty (IT) on Plant Genetic Resources for Food and Agriculture was adopted by the FAO Conference on 3rd November 2001, and will come into force once it has been ratified by 40 States. It seeks, amongst other things, to establish an access and benefit-sharing regime for plant genetic resources for food and agriculture by facilitating access to 35 food, and 29 feed crops, (the so-called "Multilateral System"); by establishing a system of fair and equitable sharing of financial benefits resulting from the commercial use of the crops covered by the Multilateral System; and by recognizing and promoting Farmers' Rights. Importantly, the IT has been drafted so that it is in harmony with the international Convention known as the Convention on Biological Diversity (CBD), and represents a significant shift away from the previously universally accepted principle that plant genetic resources are a heritage of mankind, and consequently should be available without restriction, towards the more widely accepted current position that nations have

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sovereign rights over their genetic resources, and may control and monitor access to those resources.

For mycologists carrying out biotechnology-related research, the recent international Convention that will have a particular relevance to their work is the CBD, which came into force on 29th December 1993. The CBD recognized, for the first time at an international level, the potential value of biological diversity, whether economic, environmental, or spiritual, and has introduced significant changes relating to access, control, and use of genetic resources, including fungi.

The key provisions relating to access to genetic resources are set out in Article 15 of the CBD, which seeks to strike a balance between the sovereign right of a State to regulate access to its genetic resources, on the one hand, and, on the other, an obligation to facilitate access to genetic resources "for environmentally sound uses" by other Parties. Implementation of Article 15 at a national level has been slow and, to date may not have had a significant impact upon those collecting and investigating fungi, whether for scientific or commercial uses. However, the last year has seen some major changes in the practical implementation of the CBD. In particular, at an international level, Member States to the CBD have recently adopted the "Bonn Guidelines on Access to Genetic Resources and Fair and Equitable Sharing of the Benefits Arising out of their Utilization." These voluntary, nonbinding guidelines provide framework assistance on access to genetic resources and associated benefit-sharing, and will be a useful guide to the issues that will increasingly need to be addressed before acquiring and carrying out research on fungal specimens. In addition, mycologists engaged in bioengineering should be aware of the recently agreed protocol to the CBD, the "Cartegena Protocol on Biosafety," which seeks to control the potential risks posed by cross-boarder trade and accidental releases of living modified organisms (LMOs).

In parallel with the above-mentioned debates and processes, there has been extensive discussion regarding the role that intellectual property rights can and/or should play in respect to recent developments in genetic research, and the protection of the environment. This debate is largely being played out at the international policy level under the remit of the World Trade Organization's Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS). One of the principles of TRIPS is that national laws should provide patent protection to inventions, without discrimination as to the field of technology concerned (Article 27.1). However, in response to national sensitivities about the patenting of plants and animals, WTO members agreed to include an optional exception to this principle. Article 27.3(b) of TRIPS provides that WTO Members may exclude from patentability, "plants and animals and other microorganisms, and essentially biological processes for the production of plants and animals other than nonbiological and microbiological processes." A review of this Article is currently under way in the TRIPS Council. This debate has been fuelled by allegations of inconsistency between the TRIPS Agreement and the CBD, including concerns that intellectual property rights on plant varieties erodes biological diversity, especially in agriculture.

At a more practical level, the breadth of this debate can be understood, in part, by the considerable divergence regarding the role that is already played, at a national level, by existing systems of intellectual property. For example, the legal protection of inventions is normally provided by the grant of a patent. In some countries, national legislation prohibits the patenting of any life forms. In other regions, such as the European Union, patents are excluded in respect of plants or animal varieties, or essentially biological processes for the production of plants or animals (with the exception of microbiological processes). However, in the United States, there are no such exclusions, and, in addition to plant variety rights for varieties of sexually reproduced plants, plant patents are available for asexually reproduced plants. Given that patents are only valid within the jurisdiction of the country granting the patent (there is no such thing, to date, as a single "international" patent, enforceable in a variety of different jurisdictions), all those working in the area of biotechnology, who may wish to use intellectual property rights, such as patents, to protect and promote their research, should be aware of the different national applications of intellectual property rights in relation to biotechnological inventions, and of the, often vociferous, debate at an international level of the rights and wrongs of such intellectual property rights, with a particular concern as to their potential negative effects on the protection of biodiversity.

This chapter aims to set the background to the CBD and provides an overview of the recently adopted "Bonn Guidelines on Access and Benefit-Sharing" and the "Cartegena Protocol on Biosafety," in order to draw attention to some of the main issues in the current debate in the field of intellectual property and genetic resources, and to highlight some of the practical implications of these processes and issues for individuals and institutions involved in the application of fungi for biotechnology.

2 AN INTRODUCTION TO THE CBD

The Convention on Biological Diversity, commonly known as the "CBD," is an international treaty and a framework for action for the development, largely at a national level, of legal, policy, and scientific initiatives on biological diversity. It is a relatively new convention. It was opened for signature at the Rio Earth Summit (the United Nations Conference on Environment and Development) in June 1992, and came into force on 29th December 1993. The CBD does not apply retrospectively, i.e., it does not apply to genetic resources acquired by, for instance, *ex situ* collections, before the treaty came into force on 29th December 1993.

The CBD can be ratified by individual countries and by regional organizations. As in September 2002, its membership consists of 185 countries, plus the European Union. In other words, in just over 10 years from the date when it was first opened for signature, over 90% of the countries in the

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world have demonstrated a commitment to implementing the principles set out in the CBD. An up-to-date list of ratifications can be found on the website of the CBD Secretariat: http://www.biodiv.org.

The CBD marks a milestone in international treaties. Earlier treaties tended to deal with specific aspects of biological diversity. For instance, the 1973 Convention on International Trade in Endangered Species (CITES) relates to the trade in endangered species; the Ramsar Convention, to wetlands; and the Berne Convention, to European wildlife and habitats. The scope of the CBD, however, is extremely broad, covering all components of biological diversity, from ecosystems and habitats, species and communities, to genomes and genes. Its stated objectives (Article 1, CBD) are drafted in a correspondingly broad fashion:

> the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources, including by appropriate access to genetic resources and by appropriate transfer of relevant technologies, taking into account all rights over those resources and to technologies, and by appropriate funding.

Article 2 of the CBD describes the term "biological diversity," often shortened to "biodiversity," as meaning the variability among living organisms from all sources, including terrestrial, marine, and other aquatic ecosytems, together with the "ecological complexes of which they are part." However, certain aspects of the CBD, in particular, the important provisions in Article 15 on "access" and "benefit-sharing," only apply to "genetic resources." "Genetic resources" are defined as genetic material of actual or potential value. "Genetic material" is defined as any material of plant, animal, microbial, or other origin containing functional units of heredity. "Biotechnology" is broadly described as meaning any technological application that uses biological systems, living organisms or their derivatives, to make or modify products or processes for specific use. "Technology" is simply defined as, "includes biotechnology." The terms, "microorganism" and "microbial" only appear once each in the text of the CBD, and are not defined.

Nonetheless, all those currently involved in the research of fungi, whether wholly scientific in objective, or linked to commercial, biotechnological research, and who wish to supply, or receive, authenticated, reliable biological material and associated information, clearly need to be fully aware of the implications of the CBD in their work, in particular the far reaching effects of Article 15 of the CBD: Access to Genetic Resources and Associated Benefit-Sharing.

Table 1 Summary of issues covered by Article 15 of the convention on biological diversity: Access to genetic resources

Article 15(1)	Recognizes the sovereign rights of States over their natural resources and that authority to determine access to genetic resources rests with national governments and is subject to national legislation.
Article 15(2)	Contracting Parties shall endeavor to create conditions to facilitate access to genetic resources for environmentally sound uses by other Contracting Parties and not to impose restrictions that run counter to the objectives of the Convention.
Article 15(3)	For the purposes of Articles 15, 16, and 19, genetic resources means only those resources provided by the country of origin or by parties who have acquired the resources in accordance with the Convention.
Article 15(4)	Access, where granted shall be on mutually agreed terms and subject to the provisions of this Article.
Article 15(5)	Access shall be subject to the prior informed consent of the Contracting Party providing such resource, unless otherwise determined by that Party.
Article 15(6)	Each Contracting Party shall endeavor to develop and carry out scientific research with the full participation of, and where possible in, such Contracting Parties.
Article 15(7)	Each Contracting Party shall take legislative, administrative or policy measure, as appropriate, with the aim of sharing in a fair and equitable way the results of research and development and the benefits arising from the commercial and other utilization of genetic resources with the Contracting Party providing such resources. Such sharing shall be on mutually agreed terms.

3 ACCESS TO GENETIC RESOURCES AND BENEFIT-SHARING UNDER THE CBD

3.1 An Introduction to Article 15 of the CBD

Whilst other treaties refer to access to biological material, e.g., CITES covers the import and export of certain listed species, the CBD is the only international treaty to specifically address and link "access" to genetic resources, i.e., who may collect and use those resources, with associated fair and

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equitable "benefit-sharing." Some people have called this the "grand bargain" at the heart of the Convention: the equitable exchange, on mutually agreed terms, of access to genetic resources in return for the fair and equitable sharing of benefits.

The key provisions relating to access to genetic resources are set out in Article 15 of the CBD. Article 15 seeks to strike a balance between a State's authority to regulate access to genetic resources, on the one hand, and on the other, its obligation to facilitate access to genetic resources for environmentally sound uses by other Parties. Article 15 therefore recognizes the sovereign right of states over their biological resources, and the consequent authority of national governments to determine access to genetic resources. However, Contracting Parties, i.e., signatories to the CBD, such as national governments and regional economic organizations, are also obliged to create conditions to facilitate access to genetic resources for environmentally sound uses by other Contracting Parties, and not to impose restrictions that run counter to the objectives of the Convention. Finally, Article 15 states that access shall be subject to Contracting Parties' "prior informed consent" and on mutually agreed terms that record the fair and equitable sharing of benefits from the utilization of those resources, whether those benefits are monetary or nonmonetary. The breadth of the issues covered by Article 15 can be seen in Table 1.

3.2 Practical Implementation of Article 15 of the CBD: National Legislation; Material Transfer Agreements; Codes of Conduct; and the Bonn Guidelines on Access to Genetic Resources and Benefit-Sharing

Unlike other international environmental conventions, the CBD does not lay down a particular work programme, or specific list of activities, which on signing, members become obliged to carry out. The CBD sets out a framework for action rather than a definitive statement of action. Its provisions are mostly expressed as overall goals and policies rather than precisely defined obligations. In the main, it is up to signatories to interpret these provisions according to the national situation in a particular country or region and to implement the Convention accordingly: i.e., by the development of national biodiversity action plans, national policies and guidelines, and/or national legislation regulating access to genetic resources, traditional knowledge, and associated benefit-sharing.

This means that all those who, since 29th December 1993, wish to access genetic resources in any of the 186 Contracting Parties to the Convention, must, prior to actually acquiring those resources, first ascertain how the CBD has been implemented at a national level. States are in the process of establishing the so-called "CBD Focal Points," which should be able to provide such information.* Nonetheless, as stated previously, in many jurisdictions, the situation is constantly evolving, and it is often hard to establish this information from a distance. At present, researchers would therefore be best advised to ensure that they have good local partners who can keep them informed of the actual situation on the ground and ensure that transactions are carried out in accordance with the most recently enacted laws and policies.

Furthermore, the text of the CBD is drafted in very wide terms and there are several key areas that are very vague when it comes to practical implementation. In relation to Article 15 of the CBD, what amounts to adequate "prior informed consent?" A letter, a handshake, an oral agreement, a lengthy written contract? Who precisely has authority to give such consent? What amounts to a "fair and equitable" sharing of benefits? Who is the judge of this? Many national governments are still grappling with these issues. The challenge to researchers to find answers to these questions, at the same time to develop and raise funds for, often complex, project partnerships and work programmes, may be considerable.

Nonetheless, it is clear that since the CBD came into force on 29th December 1993, all those wishing to obtain genetic resources for their research, such as fungi and associated herbarium specimens, can no longer assume that such resources are the "common heritage of mankind," freely available to all, but must follow national provisions on access, where such provisions are in place. In particular, they must clarify with those providing the samples, whether from *in situ* or from *ex situ* conditions, the terms of access and subsequent use. For instance, are the samples provided solely on the basis that they will be used for scientific research only? Can they be freely transferred to third parties? What benefit-sharing obligations might attach to the samples?

In particular, researchers should be aware that it may no longer be appropriate simply to obtain permission to collect and use biological resources from a local, in-country partner institution, such as a botanic garden or university, unless that institution has clear government authority to give such permission. These days, researchers may need to obtain a variety of permits (for collection, import, and export) and, once the specimens have arrived, will need to devise internal tracking systems to ensure that the terms of these permits are complied with. Individuals and institutions working with genetic resources acquired after the CBD came into force on 29th December 1993 are increasingly obliged to establish a "paper-trail" for all samples acquired, whether directly from *in situ* conditions, or from a third party, such as an *ex situ* collection in another country.

3.2.1 National Legislation

It is therefore becoming increasingly important for scientists to note, in this regard, that the access and benefit-sharing provisions of the CBD, not only Article 15, but also Articles

*For a complete list of CBD National Focal Point, see: http://www.biodiv.org.

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8(j), 16, and 19, are becoming a particular source of new national legislation. At a national level, over 100 countries have already introduced, or are in the process of developing, legislation and polices that will have a considerable impact on all those wishing to obtain access to genetic resources and/or traditional knowledge for biotechnological-oriented work, and may also require recipients of those genetic resources to negotiate mutually agreed terms with the provider of the resources, including agreement as to how to share fairly and equitably the monetary and nonmonetary benefits of such access. A recently compiled list of countries that are currently regulating access to their genetic resources is set out in Table 2.

Failure to ensure compliance with national legal systems could result in criminal proceedings, and prohibited or restricted access in the future for a particular individual or institution. In some countries, such as those that are members of the Andean Pact in South America (Bolivia, Colombia, Ecuador, Peru and Venezuela), failure to follow national procedures on access to genetic resources could lead to a refusal by a national intellectual property office to grant a patent based on those resources. Decision 391 of the Andean Pact on the Common Regime on Access to Genetic Resources states that access to genetic resources is subject to governmental authorization, which may consist either of a valid Certificate of Origin (for *in situ* resources), or a Material Transfer Agreement (for *ex situ* resources). Every subsequent transfer of the accessed material would have to be accompanied by this authorization. This system would link the access requirements and intellectual property regimes; patents and other intellectual property rights would only be granted on presentation of the Certificate as proof of prior informed consent.

Table 2 Regulating access to genetic resources

Regional groups, national governments, or state governments already regulating access to genetic resources to ensure prior informed consent and benefit-sharing include: the Andean Pact (Bolivia, Colombia, Ecuador, Peru, Venezuela); the Organization of African Unity (OAU); Australia (the States of Western Australia and Queensland); Brazil (at the Federal level and the States of Acre and Amapa); Cameroon; Costa Rica; the Republic of Korea; Malaysia (the States of Sabah and Sarawak); Mexico; Nicaragua; the United States of America (within Yellowstone and other national parks), and the Philippines.
Those planning to regulate access to genetic resources to ensure prior informed consent and benefit-sharing include: Albania, the member countries of the Association of South-East Asian Nations (ASEAN); Australia (the Commonwealth); Ivory Coast; Cuba; Ethiopia; Eritrea; Fiji; the Gambia; Guatemala; India; Indonesia; Kenya; Lao PDR; Lesotho; Malawi; Malaysia (at the national level); Mozambique; Namibia; Nepal; Nigeria; Pakistan, Papua New Guinea; Samoa; the Seychelles; the Solomon Islands; South Africa; Sri Lanka; Tanzania; Thailand; Uganda; Vanuatu; Vietnam; and Yemen.
Belize, China, El Salvador, Ghana, Guyana, Hungary, Iceland, Panama, the Russian Federation and Zimbabwe may also be planning to regulate access to genetic resources in the near future.

Source: Lyle Glowka, Biodiversity Strategies International, Bonn, Germany, March 2002.

The issue of the geographical origin of genetic resources in patent applications has also been addressed by the European Union in Directive 98/44/EC of the European Parliament and of the Council on the Legal Protection of Biotechnological Inventions, albeit on a voluntary basis:

> Whereas if an invention is based on biological material of plant or animal origin, or if it uses such material, the patent application should, where appropriate, include information on the geographical origin of such material, if known; whereas this is without prejudice to the processing of patent applications or the validity of rights arising from granted patents (Recital 27 of the Directive).

In contrast to Decision 391 of the Andean Pact, Directive 98/44/EC clearly states that a failure to identify the geographical origin of the biological material will not affect the validity of the patent application itself. Nonetheless, as will be addressed in more detail below, there is a growing movement towards linking the origin of genetic resources with the validity of a subsequent patent application. Mycologists should be aware that it is possible that in some countries or regions at least, it may become difficult, if not impossible, to obtain a patent on the results of genetic resource-related research, where the applicant cannot prove that the resources were originally collected in accordance with the laws of the country of origin, or where the geographical origin of those resources cannot be clearly identified. It will therefore become increasingly important to ensure that national provisions on access are followed, as and when such provisions are put in place by national governments.

3.2.2 Material Transfer Agreements

Article 15 further stipulates that the terms of the access, together with the detail of all associated benefit-sharing, must be on "mutually agreed terms." These "mutually agreed terms" tend to be recorded in written contractual agreements, often known as Material Transfer Agreements, or Access and Benefit-Sharing Agreements. These contracts can be as simple, or as complex, as the contracting parties feel is necessary and appropriate. They can range from a simple collecting permit between a National Parks Office and a researcher, to a lengthy licensing agreement involving several parties and the transfer of the genetic resources across several borders.

In this regard, and in response to a widely felt need for more information about current practices concerning

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agreements on access to genetic resources and the sharing of consequential benefits, mycologists may be interested in an initiative spearheaded by the World Intellectual Property Organization (WIPO), based in Geneva, Switzerland; namely, the establishment of an electronic, publicly accessible database of contracts related to access and benefit-sharing, in particular the intellectual property aspects of such contracts. Examples already submitted include several biotechnology-related licensing agreements. Such information will clearly be of use to all those involved in biotechnology related research and development, and will hopefully help to build the capacity of scientists and other stakeholders, particularly in those developing world, to successfully negotiate and conclude such agreements themselves. The database is available on the WIPO website at: http://www.wipo.int/globalissues/index.html.

3.2.3 Codes of Conduct

In order to comply with Article 15 of the CBD, many organizations are designing Codes of Practice and standard form agreements that set out the key terms of any access and use. For instance, the MOSAICC project (Microorganisms Sustainable Use and Access Regulation International Code of Conduct), coordinated by the Belgium Coordinated Collections of Microorganisms (BCCM) has designed a voluntary Code of Conduct for use by microbial collections, to facilitate access to microbial genetic resources and to help partners to make appropriate agreements when transferring such resources.

The Code recognizes the need for easy transfer of microbial genetic resources, and the need to monitor such transfer. Its operating principles are: (a) Identification of the origin of the resources—prior Informed Consent from the *in situ* origin should be obtained for all initial sampling. Furthermore, the *in situ* origin of the resources should always be mentioned when transfer occurs and (b) Monitored transfer of microbial genetic resources occurring under mutually agreed Material Transfer Agreements—the Code states that a Material Transfer Agreement can cover a wide variety of documents, from a short, shipping document to a more detailed, specific contract, but should contain the following information:

(i) information about the *in situ* origin,
(ii) information about both the provider and the recipient, and
(iii) mutually agreed terms relating to both the uses that may be made of the resources and the subsequent distribution of the resources by the recipient.

In addition, the MOSAICC Project partners have designed a checklist of key terms for a CBD-friendly Material Transfer Agreement: see http://www.belspo.be/bccm/mosaicc.

3.2.4 The Bonn Guidelines on Access and Benefit-sharing

One final practical issue that those working in the field of fungal biotechnology may wish to factor into future research projects is the impact of the "Bonn Guidelines on Access to Genetic Resources and Fair and Equitable Sharing of the Benefits Arising out of their Utilization" (the "Bonn Guidelines"), which were adopted by Member States to the CBD at the sixth Conference of the Parties of the CBD held in The Hague, The Netherlands, in April 2002.

These voluntary, nonbinding guidelines are not intended to be a substitute for national legislation. They have been drafted to provide framework assistance to national governments, stakeholders, providers, and users of genetic resources on access to genetic resources and associated benefit-sharing, with an overall aim of, amongst other things, reducing transaction costs. They include, for instance: (a) A list of responsibilities for providers and users of genetic resources. For example, Contracting Parties, which are countries of origin of genetic resources, or other Parties, which have acquired genetic resources in accordance with the CBD should, "ensure that all stakeholders take into consideration the environmental consequences of their access decisions," and "seek to ensure that the commercialization and any other use of genetic resources should not prevent the traditional use of genetic resources." Users of genetic resources should "only use genetic resources for purposes consistent with the terms and conditions under which they were acquired," and "maintain all relevant data regarding the genetic resources, especially documentary evidence of prior informed consent and information concerning the origin and the use of genetic resources and the benefits arising from such use," (b) A list of the steps that should be taken in the "Access and Benefit-Sharing Process." For example: overall strategy; prior informed consent, including procedures for obtaining prior informed consent; and basic requirements for mutually agreed terms, and (c) A checklist of terms and conditions that might be included in an Access and Benefit-sharing Agreement. For example, introductory provisions, such as the legal status of the provider and user of the genetic resources; access and benefit-sharing provisions, such as a description of the genetic resources covered by the material transfer agreement, a list of permitted uses, a statement that any change in use would require new prior informed consent, and the terms of any benefit-sharing arrangements; and legal provisions, such as a notice to terminate the agreement; dispute settlement arrangements; a clause on choice of law; and a clause addressing assignment of rights under the material transfer agreements: see Appendix I to the Bonn Guidelines.

As stated earlier, the Bonn Guidelines are not intended to be a substitute for national legislation. Nonetheless, given that the Guidelines provide an internationally recognized and approved set of key criteria for access to genetic resources and benefit-sharing, those involved in fungal biotechnology-related work, may wish to consider carefully the text, and its

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possible implications. For it seems likely that their recent adoption will encourage the development of corresponding national legislation, policies, and model agreements that will undoubtedly impact upon the historically free flow of genetic resources between researchers and research institutions worldwide.

4 BIOTECHNOLOGY UNDER THE CBD

The key provisions in the CBD relating to biotechnology and genetic resources are set out in Articles 16 and 19. Both of these Articles were extremely difficult to negotiate, since they address issues such as technology transfer and associated intellectual property rights that have been the subject of years of north–south debate in other *fora*. In particular, some developed countries were wary of including any language that might be interpreted as placing possible limits on the future developments of biotechnology. As a result, both articles, reflecting the significant compromises reached during the treaty negotiations, are broadly drafted, and can be difficult to interpret at a practical level.

4.1 Article 16 of the CBD

Article 16 establishes rules on access to and transfer between Parties of technologies, including biotechnologies, that are:

Relevant to the conservation and sustainable use of biodiversity. Or
Make use of genetic resources, and do not cause significant damage to the environment.

Access and transfer of technology to developing countries is to be on "fair and most favorable terms." In the case of technology subject to patents, and other intellectual property rights, access and transfer are to be provided on terms that recognize and are consistent with the adequate and effective protection of intellectual property rights. Parties will be obliged to take measures to agree mutually agreed terms with the providers of genetic resources, in particular developing countries, to ensure access to and transfer of technology that makes use of those resources, including technology protected by patents and other intellectual property rights. Further measures should be taken to ensure that the private sector facilitates access to, joint development and transfer of technology for the benefit of government institutions and the private sector of developing countries. Finally, Parties must cooperate, subject to national legislation and international law, to ensure that patents and other intellectual property rights are "supportive of, and do not run counter to" the objectives of the CBD.

Clearly, these provisions are extremely ambitious. Unfortunately, they are also open to so many different interpretations as to be almost impossible to implement at a practical level. For instance, who decides whether a particular technology will cause significant damage to the environment? And how can any particular technology, particularly one as rapidly evolving and complex, as biotechnology, be assessed, especially when an assessor may, in reality, have limited technical and administrative capacity to gather and review relevant information on technology? Furthermore, the language used in relation to intellectual property rights is sufficiently imprecise to leave open the possibility for future argument regarding the relationship between the CBD and existing intellectual property systems. Who decides whether patents and other intellectual property rights are "supportive of," and/or whether they are "counter to" the objectives of the CBD?

4.2 Article 19 of the CBD: The Cartagena Protocol on Biosafety

Article 19 is more specifically focused on biotechnology, as opposed to technology transfer generally, and addresses several aspects of biotechnology relevant to the conservation of biological diversity and the sustainable use of its components. For instance, it requires each Party to take measures to provide for the effective participation in biotechnological research activities by those providing the genetic resources, especially developing countries. Where feasible, such participation should take place in the providing country. Each Party is to take all practicable measures to promote priority access to the results and benefits from biotechnologies based on genetic resources, by the providers of these resources, especially developing countries.

Finally, Article 19 obliges Contracting Parties to:

Consider the need for a protocol dealing with issues related to the safe transfer, handling, and use of LMOs. And
Establish the basis for the bilateral provision of regulatory and impact information relating to LMOs provided to a Contracting Party.

These latter provisions have been especially productive. In January 2000, Member States to the CBD adopted the "Cartagena Protocol on Biosafety," which aims to promote the safe transfer, handling, and use of LMOs resulting from modern biotechnology that may have an adverse effect on the conservation and sustainable use of biological diversity. In brief, the Protocol establishes a procedure for ensuring that governments can signal whether or not they are willing to accept imports of agricultural commodities that include LMOs into their territory. It also establishes a Biosafety Clearing-House to facilitate the exchange of information on and experience with LMOs. In addition, commodities that may contain LMOs are to be clearly labeled as such when exported.

The Cartegena Protocol will enter into force when it has been ratified by 50 governments. As at September 2002, it has been ratified by 12 countries. As with many aspects of the CBD, once implementation becomes a reality, many of those

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currently working in the field of genetic research may need, amongst other things, to design and implement internal tracking systems to ensure that the resources on which their research is based can be followed from country to country, and to ensure compliance with associated terms of original access, use and benefit-sharing.

5 BIOTECHNOLOGY, INTELLECTUAL PROPERTY, AND GENETIC RESOURCES

As stated earlier, there is considerable discussion, at present, regarding the role that intellectual property rights, in particular patents, can and/or should play in respect to utilization of genetic resources, the protection of the environment, and the development of the biotechnology industry. For instance, in those countries where patents are available for life forms, there may be a degree of uncertainty relating to the distinction between an "invention," which can be the subject of a patent, vs. a "discovery." If a microorganism (as yet unknown) is isolated by a sophisticated process, it may be argued that such a microorganism is not an invention, but merely a scientific discovery that cannot be patented. A counter-argument might be that the isolation requires an important intervention by man using a highly sophisticated process, and that therefore the result is a solution of a technical problem, and should be protected by a patent. It may also be argued that the isolated microorganism is no different from a chemical substance that has been extracted from nature, which in many countries does amount to patentable subject matter.

Furthermore, Article 22(1) of the CBD states that the CBD will not affect the rights and obligations of any Contracting Party deriving from any existing international agreement, (which clearly includes those agreements relating to intellectual property), "except where the exercise of those rights and obligations would cause a serious damage or threat to biological diversity." Such a qualification may be relevant to those working in the field of biotechnology, since some might argue that certain aspects of biotechnology research would "cause a serious damage or threat to biological diversity."

The detail of such discussion is beyond the scope of this article, but there are two, more practical, aspects that are of particular relevance to fungal biotechnology: first, the role played by the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure; and secondly, an overview of the current trend, largely driven by the access provisions of the CBD, towards either obliging applicants for a biodiversity-related patents to disclose the origin of all genetic resources or traditional knowledge used in their claimed invention, and/or for evidence that such resources or knowledge were originally acquired legally or, as in the case in Directive 98/44/EC of the European Parliament and of the Council on the Legal Protection of Biotechnological Inventions, requesting applicants to voluntarily disclose the geographical origin of the genetic resources used.

5.1 Access to Samples of Patented Material: The Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

The Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure was concluded in order to address a very practical difficulty associated with intellectual property and microbial research. Disclosure of an invention is a generally recognized requirement for the grant of patents. Normally, an invention is disclosed by means of a written description. Where an invention involves a microorganism or the use of a microorganism, which is not available to the public, such a description will not be sufficient for disclosure. That is the reason why in the patent procedure of an increasing number of countries, it became necessary not only to file a written description, but also to deposit, with a recognized culture collection, a sample of the microorganism. In order to enable applicants to seek patents in several countries, but only to file one deposit in one specialized institution, the Budapest Treaty was concluded.

The Budapest Treaty entered into force on August 19, 1980, and still represents the only operational biotechnology-specific intellectual property treaty at a global level. It has been ratified by 55 States, including some developing countries, and three regional intergovernmental industrial property organizations, namely the European Patent Organization (EPO), the Eurasian Patent Organization (EAPO) and the African Regional Industrial Property Organization (ARIPO). The Budapest Treaty is administered by the World Intellectual Property Organization (WIPO), Geneva, Switzerland.

Under the Budapest Treaty, certain culture collections are recognized as International Depositary Authorities (IDAs). The Treaty thereby simplifies and reduces the costs of patent procedures, because one deposit with one IDA is sufficient to cover all member countries of the Budapest Treaty. It also provides certain biosafety checks and promotes technological and economic development by ensuring public access to the samples of biological material, once the relevant patent application has been published. At present, 33 IDAs in 19 countries handle the deposit and public access to biological material. The IDAs registered in the United Kingdom include the CABI Bioscience, the National Collection of Type Cultures (NCTC) and the National Collection of Yeast Cultures (NCYC).

The term "microorganism" is not defined in the Budapest Treaty, so Contracting States and IDAs are free to adopt their own definition of the term, within reasonable scientific notions. Whether an entity technically is, or is not, a microorganism matters less in practice than whether deposit of that entity is necessary for the purposes of disclosure, and whether an IDA will accept it. Thus, tissue cultures and plasmids can be deposited under the terms of the Treaty, even though they are not microorganisms in the strict sense of the word.

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The Budapest Treaty clearly establishes an important, practical procedure for any individual or institution involved in the applicability of fungi for biotechnology who may subsequently wish to submit a patent application based upon that research. A comprehensive guide to the Guide to the Deposit of Microorganisms under the Budapest Treaty, including a full list of IDAs, can be found at: http://www.wipo.int/about-ip/en/budapest/guide/index.htm.

5.2 Disclosure of the Origin of Genetic Resources, and of Prior Informed Consent, in Patent Applications

The current debate surrounding the disclosure, in biodiversity-related patents, of either the origin of genetic resources, or traditional knowledge, used in the claimed invention, or for evidence that prior informed consent was given prior to the acquisition of the resources or knowledge, has been largely driven by the access provisions set out in Article 15 of the CBD. As addressed earlier, Article 15 supports the establishment of regimes at the national level to control and regulate access to genetic resources, with the overall aim that such regimes will provide an important structural element in ensuring that the flow of genetic resources occurs in a way that optimizes the distribution of benefits to local communities, researchers, industry, and national governments. From this perspective, such regulation is of central importance to the goal of ensuring that bioprospecting is conducted in an ethical and sustainable manner.

The debate has raised a variety of difficult, practical issues, not least as to the role that the patent system can, or should, play in the enforcement of concerns that are considerably beyond the usual criteria for patentability; i.e., that the invention must be industrially applicable (useful), it must be new (novel), and it must exhibit a sufficient "inventive step" (be non-obvious).

In April 2002, the International Chamber of Commerce (ICC) published a policy paper (see: http://www.iccwbo.org/) in which, whilst supporting the objectives of the CBD and the voluntary disclosure of the source of genetic material (to the extent that it is not required by the existing patent system), it sets out its concerns regarding the establishment of any formal linkages between a requirement to disclose, and the patent system. In particular:

> Given the uncertainties as to what amounts to the "origin" of genetic material, "using the patent system to enforce conditions of disclosure would likely inhibit both the beneficial use of the resources and any sharing of benefits. Such requirements in our view would conflict with Article 15 of the CBD, which requires signatory states to facilitate access." And
>
> Given the fact that the CBD is being implemented by different countries at different rates and in different ways, "there should be no question of a general requirement for all national Patent Offices to require evidence of (prior informed consent) PIC when a patent application referring to biological resources is filed. It would be disproportionate. Further, the requirements of countries restricting access will differ one from another. It would be difficult or impossible to construct clear general rules to which companies must conform. There is fundamental lack of clarity as to when PIC is required. Suppose the resource has come via a third country? Suppose the resource is common and widely distributed? Suppose the resource does not make a fundamental contribution to the invention patented? Where are lines to be drawn, and what principles are to be applied in drawing them?"

At the sixth meeting of the Conference of the Parties to the CBD in The Hague in April 2002, there was considerable discussion regarding whether, as a condition of patentability, patent applicants should disclose the origin of biological material on which patents are filed, and whether prior informed consent should be demonstrated. The result of this discussion was that the CBD invited the World Intellectual Property Organization (WIPO), the specialized United Nations Agency responsible for the promotion of intellectual property protection, to carry out a technical study on methods consistent with obligations in treaties administered by WIPO for requiring the disclosure within patent applications of, amongst other things:

> Genetic resources utilized in the development of the claimed inventions.
>
> The country of origin of genetic resources utilized in the claimed inventions.
>
> Associated traditional knowledge, innovations, and practices utilized in the development of the claimed inventions.
>
> The source of associated traditional knowledge, innovations, and practices. And
>
> Evidence of prior informed consent.

The study will be published for the seventh meeting of the Conference of the Parties to the CBD in 2004. It should be of particular interest to all those involved in access to genetic resources and subsequent biotechnology-related research, since any linkages between access to resources and the patent system may have significant practical implications. In particular, those carrying out biotechnology-related work, whether as a provider or a recipient of genetic material, may need to consider, in some detail, the terms under which they acquire or supply genetic resources. As stated earlier, institutions should seriously consider the establishment of internal systems to ensure, amongst other things, that an adequate record is kept of the original terms of acquisition, including the original source of the material, and that evidence of any prior informed consent is carefully retained, where such consent

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is required under national legislation in force at the time of access. If such systems are not in place, researchers may find that they carry out expensive and time-consuming research only to discover that they run into difficulties when trying to protect their invention, using the patent system.

6 CONCLUSIONS

The rules governing access to genetic materials, such as plants and fungi, have changed dramatically in the last decade. Previously, genetic resources were usually viewed as the "common heritage of humankind," and access arrangements often were informal. With the advent of the CBD, the sovereign rights of States over their biological resources has been recognized. The authority to determine access to genetic resources now rests with national governments and arrangements for access and associated benefit-sharing are increasingly being regulated by access legislation, and recorded in written contractual agreements.

Furthermore, the interaction between the intellectual property system, the utilization of genetic resources, and protection of the environment has been the subject of increasing international debate. For some, the relationship between these issues is seen as providing a mechanism that may lead to the exploitation and degradation of the environment. For others, there are potential benefits, for both the economy and the environment, of the practical application of intellectual property rights for assisting in the development of domestic industries and research in areas such as biotechnology.

At present, there are no easy or rapid answers. However, those involved in microbial research, such as the application of fungi in the field of biotechnology, need to be aware that the international debate is beginning to have practical implications at a national or local level, and that any future project planning and research will need to take these implications into consideration. The "Bonn Guidelines on Access to Genetic Resources and Fair and Equitable Sharing of the Benefits Arising out of their Utilization," and the current debate regarding whether or not the origin of genetic resources, and evidence of prior informed consent, should be disclosed in patent applications, as a condition of patentability, or simply on a voluntary basis, are two of the more significant recent developments in this field.

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