Pathogenesis of Bacterial Infections in Animals

Fourth Edition

EDITED BY

Carlton L. Gyles
John F. Prescott
Glenn Songer
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J. Glenn Songer, and Charles O. Thoen

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Preface

The fourth edition of *Pathogenesis of Bacterial Infections in Animals* captures the fascinating and rapid developments in understanding of the mechanisms of virulence of the major bacterial pathogens of animals. The book is the product of the efforts of 74 authors, all experts in the field on which they have written. The authors come from 13 countries: Australia, Brazil, Belgium, Canada, Germany, Ireland, Israel, Mexico, Norway, Switzerland, the United Kingdom, the United States, and the West Indies.

There have been a few changes in the chapters that were present in the third edition. We omitted the chapters on *Shigella* and *Erysipelothrix rhusiopathiae*. We decided against a chapter on *Shigella* because its primary importance is as a human pathogen and on *E. rhusiopathiae* because there is a very limited literature on mechanisms of disease by this organism. This is likely because effective vaccination and control of the disease have reduced the opportunities for funded research on pathogenesis. We added a chapter on subversion of the immune response by bacterial pathogens, because this subject is an important and underemphasized aspect of pathogenesis of many bacterial infections and there have been new insights into the mechanisms employed by bacteria to thwart the host immune response. We presented *Pasteurella* and *Mannheimia* as separate chapters to reflect important differences in pathogenesis in these two genera.

One of the challenges in preparing the book was the question of adequate attribution of research findings. Given the volume of literature on each pathogen, there was a danger that too much of the book would be a listing of references. However, the ready availability of excellent modern bibliographic search systems means that the book does not have to serve as the only source of references on pathogenesis of bacterial infections in animals. We therefore made the decision to curtail the number of references, recognizing with regret that not all researchers who made contributions to the literature will be recognized.

This book is possible because a vast number of people believe in sharing their knowledge and perspective with the rest of the bacterial pathogenesis community. It is based on the work of hundreds of researchers, whose ideas, innovation, and research skills have produced the body of knowledge on which we draw. The authors of the chapters in this book have presented captivating stories of bacterial pathogenesis based on their work and that of their colleagues. Several authors created illustrations that are valuable adjuncts to description of pathogenesis. In many cases, publishers and colleagues kindly gave permission for the use of illustrations in the book.

Although molecular pathogenesis is a major aspect in almost every chapter, authors have been careful to place pathogenesis in its broader context. The extent of this context varies from one pathogen to another, partly because bacterial diseases develop under widely varying circumstances and partly because the extent of knowledge of the circumstances varies considerably. As in the previous editions, we have presented both the overview of

pathogenesis, including relevant events that occur in the herd or flock and its environment, and activities that take place at the cellular and molecular levels.

Finally, we wish to thank our publishers who have been extremely helpful in transforming the writings of 64 individuals into a beautiful book. Special thanks go to Erin Magnani, the production editor; Nancy Simmerman, editorial assistant; and Justin Jeffryes, commissioning editor.

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Pathogenesis of Bacterial Infections in Animals

Fourth Edition

1
Themes in Bacterial Pathogenic Mechanisms

C. L. Gyles and J. F. Prescott

INTRODUCTION

The speed of progression of our understanding of pathogenic bacteria and their interactions with the host at the molecular level is providing novel insights and perspectives on pathogens and pathogenicity at an almost overwhelming rate. Such information and insights are of fundamental value in designing better and unprecedented ways to counter infectious diseases. For example, studies on the use of drugs that jam quorum sensing communication systems have shown promise that this approach may be an effective method of preventing virulence regulons from being activated (Hentzer et al. 2003; Rasko et al. 2008). In a recent study, Rasko et al. (2008) identified a novel compound (LED209) that blocks the bacterial histidine sensor kinase, QseC, which is found in several gram-negative bacterial pathogens and is required for expression of certain virulence genes. These authors have shown that LED209 is nontoxic to mice and protected mice against death due to *Salmonella* Typhimurium or *Francisella tularensis*. Rasko and coworkers (2008) have noted that, unlike antibiotics, this anti-virulence approach does not threaten the life of the bacteria and may therefore not exert a selective pressure that selects for resistant organisms. However, if this method is a threat to a critical niche for these bacteria, it could also have a selective effect.

Although an overview of the basic themes in bacterial pathogenic mechanisms provides a conceptual skeleton for the extensive details of individual pathogens given in later chapters, understanding of virulence and pathogenicity is changing rapidly. The fundamental concepts have withstood the test of time fairly well, but new knowledge has brought the complexities of host–pathogen interactions into sharper focus and has identified nuances that had not been recognized *previously* (Finlay and Falkow 1997; Bhavsar et al. 2007). Although more is understood about bacteria, especially through the application of genome sequencing and related technologies, bacterial infections seem to be increasing and changing, in particular those associated with increased antibiotic resistance, driven by exposure to more powerful antibiotics. Numerous anthropogenic activities including antibiotic use at both therapeutic and subtherapeutic concentrations may be driving bacterial evolution and the selection of pathogens adapted to changed circumstances (Chopra et al. 2003; Davies et al. 2006). Against the background of stunning advances in technologies, there is increasing recognition of the poor general application of well-established simple infection control techniques such as hand-washing to reduce the burden of infection in people and in animals in clinical settings. The fight against bacterial infections requires constant vigilance and disciplined use of hard-earned knowledge, not simply the application of new technology.

BASIC STEPS IN PATHOGENESIS CONTINUE TO PROVIDE A SOUND FOUNDATION

The basic steps in the establishment of infection by a bacterial pathogen are:

1. attachment or other means of entry into the body;

2. evasion of normal host defenses against infection;
3. multiplication to significant numbers at the site of infection and/or spread to other sites;
4. damage to the host, either directly or through the nonspecific or specific immune host response to the bacterium;
5. transmission from the infected animal to other susceptible animals, so that the infection cycle can continue.

As would be expected for carefully regulated systems, the infection process is a dynamic continuum rather than a clear series of steps, but breaking it down into progressive steps allows ease of understanding.

Pathogen Association with the Host

Successful colonization of the skin or a mucosal surface of the host is usually the first prerequisite of the infectious process. Some organisms need to employ motility and chemotaxis as well as resistance to acid and bile in order to reach their target host cells. Initial contact between bacterial pathogen and host cell is usually mediated by fimbrial or nonfimbrial adhesins on the bacterial surface (Kline et al. 2009). Binding may result either in extracellular colonization or in internalization of the pathogen. The adhesins bind to specific host cell surface receptors, and both host and organ specificity of infection may be determined by differences among animals in cellular, receptors for the bacterial adhesins. For example, the *Listeria monocytogenes* adhesion molecule internalin A (InlA) promotes uptake of the bacterium into intestinal epithelial cells by binding to E-cadherin. InlA binds to human and rabbit E-cadherin and causes disease in these species; however, it fails to bind to mouse E-cadherin and so does not cause disease in mice. Interestingly, Wollert et al. (2007) recently showed that by making two substitutions in InlA they could increase the binding affinity to mouse E-cadherin by 10,000-fold and thereby establish experimental infection in mice. The researchers noted that newly emerging diseases may arise by similar naturally occurring mutations.

As many receptors are developmentally regulated, age specificity may also be determined by the receptor to which a pathogen binds. This is seen in K99 (F5) pili of porcine and bovine enterotoxigenic *Escherichia coli* (ETEC), which bind to the intestinal epithelium of neonatal animals, and in F18 pili of porcine ETEC, which bind to the intestinal epithelium of recently weaned pigs.

Bacterial pathogens, including those associated with wound infections, may bind to extracellular matrix molecules such as fibronectin, collagen, laminin, or other proteins possessing RGD (Arg-Gly-Asp) sequences for binding of eukaryotic cell membrane integrins. Bacteria may use "invasins" to mediate their uptake into nonprofessional phagocytic host cells after attaching to molecules on the cell surface and activating host cell signaling to facilitate their entry, often through host cell cytoskeletal rearrangement (Galán and Cossart 2005). An excellent example of this is found in the adherence to and invasion of M cells by *Yersinia enterocolitica* and *Y. pseudotuberculosis*. The outer membrane protein invasin produced by these bacteria binds to β1 integrin on the surface of M cells and triggers uptake of the bacteria in a zipper-like internalization process (Hauck 2002). This entry provides the bacteria with access to the lymphoid tissue below, and to draining lymph nodes, in which the bacteria are well equipped to multiply.

Facultative intracellular pathogens may deliberately target macrophages, for example by entering through complement- or other lectin-binding receptors and thus avoiding the oxidative burst that might otherwise kill them. Remarkably and, at first sight, paradoxically, the safest place in the body for these organisms, which subsequently interfere with normal macrophage phagosome maturation, is actually a macrophage.

Pathogen Multiplication and Evasion of Host Defenses

After initial association with the host, bacterial pathogens need to evade host defenses and to multiply to numbers sufficient for the infection to be self-sustaining rather than to be aborted by the host response. The "defensins" involved in the evasion–multiplication process can be divided into those involved in defense against innate immune mechanisms and those involved in defense against specific immune mechanisms.

Innate immunity can be overcome in a wide variety of ways (discussed throughout the book, in particular Chapter 2). The lack of available iron that restricts the growth of many bacteria within the body is an important defense mechanism as iron is critical for iron-containing cofactors for enzymes

required for primary and secondary bacterial metabolism. This limitation is often overcome by the iron-acquisition systems of pathogens. Recognition of the importance of iron acquisition by pathogens has led to a recent focus on inhibiting siderophores in the development of novel antibacterials (Miethke and Marahiel 2007). This is part of an approach that recognizes that inhibition of bacterial growth alone as a screening approach to antibacterial-drug discovery will result in numerous potential important pathogen targets being missed (Davies et al. 2006).

Many organisms, particularly those that cause septicemia and pneumonia, have prominent, usually carbohydrate, capsules that help the organism resist phagocytosis in the absence of antibodies. Some capsules mimic host matrices so that the organisms are unrecognized by phagocytes. The lipopolysaccharide molecules of some gram-negative bacteria can protect them from the membrane attack complex of complement or from the insertion of antimicrobial peptides. Some bacteria such as streptococci can break down complement components through C5a peptidase or other proteases. Other bacteria may destroy or impair phagocytic cells through their leukocidins such as the RTX (repeats in the structural toxin) toxins, or enable bacteria to survive inside phagocytes through enzymes such as superoxide dismutases or catalases.

Acquired immunity can be overcome in several ways. These include the ability to degrade immunoglobulins with enzymes such as the IgA proteases of *Histophilus somni*, or the ability to alter the antigenicity of cell surface components such as fimbriae or outer membrane proteins. Bacterial superantigens can dramatically up-regulate certain T cell subsets with specific V_β regions, which may result not only in a "cytokine storm," which confuses the immune system, but also in the deletion of these cells from the immune repertoire. In ways that are not well understood, some bacteria, such as *Rhodococcus equi*, may modulate the cytokine response to infection so that an ineffective Th2 rather than effective Th1-based immune response leads to development of disease. The role of "modulins" in diverting the host immune response is far less well understood for bacteria than for viral infections.

Pathogen Damage to the Host

Bacterial damage to the host is usually essential for immediate or long-term acquisition of the nutrients that the bacterium needs to thrive and to continue its pathogenic lifestyle. Infection does not always lead to disease, which is only one of the possible outcomes of bacteria–host interaction. Other outcomes include commensalism and latency.

Among the wide variety of "offensins" produced by bacteria are many different types of toxins. Toxins can be classified in different and not fully satisfactory ways, although that based on activity is the most logical (Wilson et al. 2002). Type I toxins, the membrane-acting toxins, bind to cell surface receptors to transduce a signal that results in the activation of host cell pathways, leading to aberrant cell metabolism. Examples in *E. coli* include the heat-stable enterotoxin a STa, which binds to the receptor for guanylyl cyclase, resulting in hypersecretion due to excessive levels of cyclic guanosine monophosphate (cGMP), and the cytotoxic necrotizing factor (CNF) toxins, which activate Rho guanosine triphosphatases (GTPases), resulting in cytoskeletal rearrangements. Other examples include the *Bacillus anthracis* edema factor (EF), the *Pasteurella multocida* toxin (PMT), and the exoenzyme S (ExoS) toxin of *Pseudomonas aeruginosa*. The superantigens fall into this class. Type II toxins, the membrane-damaging toxins, include the membrane channel-forming toxins using the β-barrel structure (e.g., *Staphylococcus aureus* α-toxin), channel-forming toxins involving α-helix formation, the large range of thiol-activated cholesterol-binding cytolysins, and the RTX toxins. Type II toxins that damage membranes enzymatically include the phospholipases of many bacteria. Type III toxins, the intracellular toxins, are toxins that enter and are active within the cell. These are often active-binding (AB) two-component toxin molecules. Examples include the adenosine diphosphate (ADP)-ribosyl transferasesf (e.g., the *E. coli* heat-labile enterotoxin [LT]), the N-glycosidases (e.g., the Shiga toxins), the adenylate cyclases (e.g., the *Bordetella bronchiseptica* adenylate cyclase toxin), and the metalloendoproteases of the clostridial neurotoxins.

Tissue damage and impairment of host function is often due to the inflammatory response mounted by the host in response to infection with a bacterial pathogen. Sepsis represents an extreme case in which hyperresponsiveness to lipopolysaccharide (LPS) and/or other host signaling molecules unleashes an excessively strong inflammatory response resulting in vascular damage, hypotension, and multiple organ damage. The inflammatory response

mounted by the host may also provide a point of entry for certain invasive enteric pathogens, such as *Shigella dysenteriae*. This organism carries a virulence plasmid-encoded homologue of the *msbB* gene in addition to the chromosomal copy, and it has been suggested that this may ensure complete acylation of lipid A and production of highly stimulatory LPS. The massive leukocyte infiltration between epithelial cells promotes invasion by the pathogens (D'Hauteville et al. 2002). A similar arrangement for the *msbB* gene exists in *E. coli* O157:H7.

Pathogen Transmission from the Host

Although not often considered in a discussion of bacterial pathogenesis, a crucial feature of bacterial pathogens is their ability to use their pathogenic nature to ensure their further transmission from the host, either back into their environmental reservoir or directly to other susceptible hosts. Depending on the infection, further transmission to animals may be immediate or may even involve decades.

An important aspect of transmission involves bacterial infections of animals that are important primarily because of the transmission of organisms from animals to humans. In some cases, as with Shiga toxin-producing *E. coli* (STEC) O157:H7, the bacteria are normal flora in the intestine of animals, where they do not cause disease; however, they do induce severe disease following transmission to humans. A similar situation exists for *Campylobacter jejuni and* most serotypes of *Salmonella* in poultry. Efficient transfer from their reservoir hosts to their accidental host occurs directly through contamination of foods of animal origin and indirectly through fecal contamination of water and the environment.

CONCEPTS OF VIRULENCE ARE BEING REFINED

Bacteria cause disease by a variety of virulence mechanisms in a highly complex process that usually involves penetrating the host's protective barriers, evading deeper host defenses, multiplying to significant numbers, and damaging the host, leading to escape from the host to continue the cycle. Although this concept of virulence is well established, the resurgence or emergence of infectious diseases in humans in recent years because of changes in host susceptibility (AIDS, immunosuppressive drugs, hospital-acquired infections) emphasizes the importance of host factors in determining the outcome of encounters with microbes. Many people now die in hospitals from infectious agents that are not pathogens in healthy people. A parallel situation exists in many small animal hospitals, especially in intensive care units. Similarly, the ability of some bacteria rapidly to develop or acquire antimicrobial resistance and then to emerge as significant problems in hospital or community settings emphasizes the importance of environmental factors in determining the outcome of infection. Virulence does not occur in a vacuum; it is contextually dependent. In this case, antibiotic use in hospitals may remove the inhibitory effects of the normal microbial flora in reducing colonization by exogenous, resistant, bacteria. Furthermore, bacterial pathogens themselves may carry genes for bacteriocins that are sometimes linked to virulence genes or bacteriophages that possess virulence genes, which are an important part of their success as pathogens. Selective pressures other than just interaction with the host may exert profound influence on the evolution of pathogens (Brown et al. 2006).

The impact of infection on the evolution of animal hosts can generally only be speculated upon but may be profound. The evolution of hosts and the pathogens that exploit them are inexorably linked (Brown et al. 2006). For example, the target of the *Vibrio cholerae* toxin (CT) and *E. coli* heat-labile enterotoxin (LT) is the cystic fibrosis transmembrane conductance regulator (CFTR) protein, whose response to CT leads to fluid outpouring in the intestine. The CFTR protein is necessary for fluid secretion in the intestine and in airways, and intestinal tissue from patients with cystic fibrosis fails to respond to CT. It has been suggested that the defects in the CFTR gene that provide resistance to cholera may have led to the maintenance of defective genes in the human population and the high frequency of the delta F508 mutation (1 in 25); individuals who are homozygous for this mutation develop cystic fibrosis. Interestingly, recent evidence suggests that this mutation may also provide protection against infection with *Salmonella* Typhi (Pier et al. 1998). The historical association of pathogens and their hosts, and the coevolutionary nature of this relationship, are also part of the host–pathogen–environment triad that determines the outcome of an infection.

Earlier definitions of virulence often derived from older studies of classic bacterial pathogens ("Koch's postulates"), many of which have been controlled by immunization, hygiene, or antimicrobial drugs. These definitions were markedly updated (Falkow's [1988] "molecular Koch's postulates") but were still largely pathogen-centered and focused on a narrow

range of virulence determinants such as exotoxins of *Corynebacterium diphtheriae* and A more recent theme, even ignoring host and environmental interactions with the pathogen as determinants of disease, has emphasized that bacterial virulence is multifactorial, involving not only "true" or "essential virulence genes" that are directly responsible for host damage, but also "virulence lifestyle genes" that regulate essential virulence genes or are otherwise required for their expression, secretion, or processing, as well as " virulence lifestyle genes" that allow bacteria to colonize the host, evade host defenses, use host factors for survival, or survive intracellularly (Wassenaar and Gaastra 2001). An analogy is to a gun: the bullets can be considered the true virulence genes, the gun can be considered the virulence-associated genes, and the criminal can be considered the virulence lifestyle genes. Clearly, inactivation of any of these three elements will stop the bullets killing a victim, but ultimately it is the bullets that kill. Recognition of the distinction of these different elements will prevent some of the potential confusion that faulty interpretations of modern experimental methods produce. Bacterial virulence is thus more clearly than ever recognized as the truly complex, dynamic, changeable, and often surprising phenomenon that it is.

This view of bacterial virulence highlights the survival and successful further spread of bacteria under potentially adverse conditions in the ecological niche(s) into which they have been introduced or to which they have adapted, and all the complexity that successful survival implies. From this perspective, antimicrobial resistance genes may contribute to virulence as they are virulence lifestyle genes that contribute to survival in antibiotic-containing environments.

HOST–BACTERIA COMMUNICATION IS CRITICAL

It has long been recognized that the outcome of infection is dependent on complex multistep processes involving host, pathogen, environment, and their interactions. Nonetheless, the tendency has been for researchers to tackle problems of pathogenesis primarily by investigation of virulence attributes of the pathogen. One of the outcomes of this approach is that we now have an impressive catalog of virulence genes of bacterial pathogens, but we have a long way to go in understanding issues of regulation, timing, cross talk, and interplay with host structures and physiology. In recent years, researchers have sought to redress this imbalance, and we have seen numerous investigations of pathogens in either their natural host environments or in *in vitro* settings that seek to simulate aspects of the *in vivo* environment. It is therefore not surprising that a major theme in pathogenesis research is that communication among bacteria, host, and environment is a critical aspect of pathogenesis. Studies in this field have led to a new branch of microbiology, namely cellular microbiology, which investigates bacterial signal transduction as a tool for characterizing host signaling pathways.

Bacteria have an astounding ability to sense their environment and rapidly to respond to it. Bacteria–host–environment communication systems that are important in pathogenesis may involve combinations of bacterial type III secretion systems (TTSS), type IV secretion systems, host cell cytoskeletal rearrangement, quorum sensing, two-component regulatory systems, and stress responses. Studies of TTSSs have identified a conservation of the secretion apparatus and a remarkable diversity in the effector functions mediated by the systems in extensively investigated bacterial pathogens such as *Salmonella*, *Shigella*, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Yersinia*. The effectors of the TTSSs are virulence factors that interact with specific host cell structures and factors that set off complex host cell pathways (Gruenheid and Finlay 2003).

Bacteria need to be aware of their environment so as to know when to deploy their virulence genes. Cues to bacterial location are as diverse as temperature, pH, growth phase, nutrient availability, oxygen levels, ion concentrations, and quorum sensing molecules, or combinations of these cues. Depending on the environment, some virulence genes may be upregulated while others are downregulated only to reverse when the environment changes. The regulation of virulence is complex, with several regulators often controlling the expression of a particular virulence gene and with coordinate regulation of genes whose products are required under the same circumstances. The practical application of knowing how virulence genes are regulated is that regulation is a potential and underexploited target for novel synthetic or natural inhibitory molecules.

An interesting study by Hentzer and colleagues (2003) showed in a dramatic fashion how sabotage of the bacteria–host communication system might be used to attenuate bacterial virulence. These

researchers targeted the quorum sensing circuits of *P. aeruginosa*, which are known to regulate critical virulence factors in this organism. The researchers demonstrated that a synthetic furanone compound, C-30, was antagonistic toward the quorum sensing systems of *P. aeruginosa.* The researchers then used transcriptome analysis to identify 93 genes that were more than fivefold affected by application of C-30 to a culture of *P. aeruginosa*, and noted that 30 of 85 C-30-repressed genes were quorum sensing-controlled major virulence factors. Additional experiments showed that growth of *P. aeruginosa* biofilms in the presence of C-30 resulted in biofilms that had lost their resistance to sodium dodecyl sulfate (SDS) and to tobramycin. Subsequent experiments demonstrated that C-30 was effective against *P. aeruginosa* in the lungs of infected mice: those mice that were treated had lung bacterial contents that were, on average, 1/1000th those of untreated mice. Encouraging data obtained during the study included observations that the effect of C-30 was highly specific as it targeted only the *las* and *rhl* quorum sensors, and was effective against both planktonic and biofilm cultures. The authors suggested that an attack on expression of virulence was unlikely to create pressures for resistant mutants to develop.

Further studies that pursue this "antipathogenic drug principle" have used computer-assisted structure-based virtual screening to identify other compounds that inhibit quorum sensing-regulated gene expression (Yang et al. 2009).

New regulatory signals that are critical for virulence expression are being identified. For example, EHEC O157:H7 expresses the colonization genes encoded by the locus for enterocyte effacement (LEE) in response to a quorum sensing regulatory molecule that was initially considered to be autoinducer 2 (AI-2; Sperandio et al. 2001) but has now been shown to be a new autoinducer called AI-3 (Sperandio et al. 2003). Both AI-2 and AI-3 require LuxS for their synthesis. Interestingly, the mammalian hormones epinephrine and norepinephrine had the same effect as AI-3 in activating the LEE-encoded genes (Sperandio et al. 2003). Furthermore, either exogenous AI-3 or epinephrine could activate the LEE genes in a *luxS* mutant, and epinephrine antagonists could block this activation. These data suggest that AI-3 and epinephrine may use the same bacterial signaling pathway in cross-talk between host and pathogen. Epinephrine and AI-3 act synergistically, and it has been proposed that EHEC in the intestinal lumen respond to AIs produced by commensal and pathogenic bacteria, resulting in activation of motility. Once the bacteria arrive at the host epithelium, epinephrine stimulates expression of the LEE genes, thereby allowing EHEC to attach to the intestine (Kendall et al. 2007). Evidently, factors that influence the epinephrine/norepinephrine content in the intestine may play a significant role in expression of LEE and the virulence genes in EHEC. In *P. aeruginosa*, the quorum sensing signal can act directly on host cells to stimulate production of the pro-inflammatory cytokine IL-8 (Smith and Iglewski 2003). It will be interesting to see whether in an analogous fashion AI-3 has a direct effect on host intestinal epithelial cells.

Signaling that affects host apoptosis pathways is a common aspect of pathogenesis of bacterial diseases. Bacterial products that have been shown to induce apoptosis include outer membrane proteins, LPS, lipomannans, lipoarabinomaannans, lipoproteins, porins, and certain protein toxins. Macrophages, by their possession of receptors for conserved bacterial surface components such as LPS and lipoprotein, are highly vulnerable, as apoptosis may be triggered by reactions with these receptors.

Apoptosis is a common feature of pathogenesis in a wide range of pathogens including *Campylobacter*, *Chlamydia, Escherichia*, *Listeria*, *Mycobacterium*, *Pseudomonas*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, and *Yersinia.* Apoptosis may provide benefits to the host by curtailing the primary immune response, thereby limiting damage due to excessive release of cytokines and destructive neutrophil enzymes. On the other hand, it may be of value to the parasite by destroying host defense cells such as macrophages, thereby promoting bacterial invasion of tissues and prolonging infection.

Bacteria are able to induce apoptosis by stimulating proapoptotic molecules or inhibiting anti-apoptotic molecules (Grassme et al. 2001). Direct activation of pro-aptototic signals is seen in infection by *Salmonella*, *Shigella flexneri*, and *Staphylococcus aureus*. *Shigella flexneri* uses its TTSS to inject M cells with IpaB, which binds to caspase 1, and activates its proteolytic function, thereby inducing apoptosis. Simultaneous release of IL-1 by the M cells attracts neutrophils and widens the junction between epithelial cells, thus facilitating access to the basolateral surface of epithelial cells, a site that is vulnerable to invasion by *S. flexneri*. *Salmonella*

Typhimurium also uses its TTSS to inject SipB, which activates caspase 1. The mechanisms by which *S. aureus* induces apoptosis are less well understood, but they involve activation of caspases, JUN-N-terminal kinases (JNK), and acid sphingomyelinase. Alpha toxin has been identified as the staphylococcal protein that initiates apoptosis.

A TTSS also features in induction of apoptosis in macrophages by *Yersinia*. These bacteria both activate pro-apoptotic pathways and inhibit anti-apoptotic pathways. Injection of YopP (*Y. enterocolitica*) or YopJ (*Y. pseudotuberculosis* and *Y. pestis*) into macrophages activates caspases and inhibits activation of mitogen-activated protein (MAP) kinase and MAP kinase kinases as well as nuclear factor κB (NfκB). Inhibition of the translocation of NfκB into the nucleus causes suppression of the production of TNF-alpha, a stimulator of apoptosis.

The translocated intimin receptor (Tir) of EPEC has been identified as a TTSS-delivered molecule that can trigger apoptosis in cultured epithelial cells (Malish et al. 2003). Tir was localized to the mitochondria, but its mechanism of induction of apoptosis is unknown. Shiga toxin injures host vascular endothelial cells by an apoptotic mechanism (Erwert et al. 2003). This activity is dependent on the A subunit of the toxin and is effected by inhibition of McI-1, a member of the Bcl-2 family of antiapoptotic proteins. *P. aeruginosa* depends on its TTSS to upregulate expression on the surface of epithelial cells of CD95/CD95 ligand, a receptor/ligand pair that stimulates apoptosis through signaling of caspases.

The end result of apoptotic changes varies in the different infections. For example, apoptosis triggered by *P. aeruginosa* appears to be critical for the host defense against lung infection, presumably by promoting phagocytosis of bacteria that are packaged in apoptotic bodies (Cannon et al. 2003). The muted inflammatory response involved in apoptosis may also be beneficial to the infected tissue and the host. In the intestine, the elimination of invading bacterial pathogens by an increased rate of apoptosis of intestinal epithelial cells may be an important mechanism for the elimination of pathogens such as EPEC (Malish et al. 2003). At the same time, deeper tissues may become vulnerable to invasion by intestinal bacteria. In contrast, apoptosis by *S. flexneri* is a critical aspect of infection by the bacterium. It is needed for invasion of the intestinal epithelium.

Host cells also have elaborate mechanisms for identifying conserved bacterial structures and relaying this information to pathways that respond to the presence of bacteria. Pattern recognition receptors (PRRs) on the surface of innate immune cells permit the recognition of infectious agents through their possession of pathogen-associated molecular patterns (PAMPs) such as LPS, lipoproteins, peptidoglycans, and DNA with unmethylated CpG motifs. Included among the PRRs are the Toll-like receptors (TLRs), which are signal transduction proteins that, among other actions, trigger the secretion of cytokines through activation of the transcription factor NfκB. Signaling by TLRs occurs primarily through the adaptor molecule MyD88. Recently, another adaptor molecule (Trif) was shown to be required for signals leading to the production of interferon-β following activation of TLR3 or TLR4. TLR3 detects double-stranded RNA; TLR4 recognizes LPS; and TLR2 recognizes lipoproteins, peptidoglycans, and lipoteichoic acid. Flagellin binds to TLR5 and causes the release of IL-8 from intestinal epithelial cells. Interestingly, TLR5 is expressed on the basolateral and not on the apical surface of intestinal epithelial cells so that the alarm is sounded only when bacterial invasion has occurred or bacterial products have reached this site. CpG–DNA interacts with TLR9, which is located intracellularly rather than at the cell surface. Internalization of CpG–DNA and endosomal maturation are necessary for activation of TLR9 (Ahmad-Nejad et al. 2002).

The TLRs help to link the innate immune response with the acquired immune response, as macrophages and dendritic cells that contact pathogens become activated, causing the upregulation of co-stimulatory cell surface molecules as well as class I and II major histocompatibility complex (MHC) molecules. Differential expression of TLRs on the various types of cells of the innate immune system and differences in the signals that are generated allow for a system in which the type of pathogen that is encountered is met with the appropriate Th1 or Th2 response. (Wagner 2002). Innate immune responses that occur following binding of the pathogen to a TLR include killing of the pathogen through antimicrobial compounds such as nitric oxide in macrophages and antimicrobial peptides at the surface of epithelial cells (Sieling and Modlin 2002). Adaptive immune responses are influenced through the activation of B cell proliferation, release of chemokines, and adjuvant effects of the PAMPs.

PATHOGENESIS IN THE POST-GENOMIC ERA

The enormous influx of information from genome sequencing is revolutionizing the science of pathogenesis, ranging from understanding the most basic aspects of gene content to elucidating the regulatory networks of virulence gene expression, to investigating the global patterns of host response to infection (Medini et al. 2008). Examining differences in specific genes between a pathogen and a closely related nonpathogen, or between the parent and its offspring with a specific null mutation, has been a valuable approach for identifying virulence genes. The rate of recognition of potential virulence genes is increasing dramatically as complete or almost complete genome sequences are now available for thousands of bacteria, and genomic data and microarray analysis are now frequently combined rapidly to identify hundreds of potential virulence factors simultaneously. However, these potential virulence factors will need to be tested individually to assess their roles in virulence.

The immensity and complexity of data that need to be analyzed are almost overwhelming (Medini et al. 2008). Initially, genomics brought us the concepts of the core genome (encoding the basic aspects of the bacterial biology) plus the dispensable and strain-specific genes for an isolate. We have now moved beyond this and the pan-genome (the genetic information of a bacterial species) and the metagenome (the genetic information of a community of bacteria in a specific environmental niche) have moved from theoretical constructs to reality.

Functional genomics can be used to investigate the transcriptome under specific conditions. Data from transcriptome studies are beginning to lead to a better understanding of memberships in virulence regulons and to the identification of the complex environmental cues that modulate virulence expression. Ideally, bacterial mRNA collected from infected tissues would be examined. However, relatively low numbers of bacteria in most infected tissues, relatively small amounts of bacterial compared with host RNA, and instability of bacterial mRNA make this approach impractical for most infections. Hence, it has been necessary to use amplification methodologies such as selected capture of transcribed sequences (SCOTS) or *in vitro* conditions to simulate the *in vivo* setting. One of the challenges in these studies is to accurately simulate the host microenvironment. Currently, it is common for only one or two aspects of that environment to be examined in simulations, for example temperature, iron concentration, and pH. It is likely that much more complex simulations will be attempted in the future.

Comparative genomics involving comparison of open reading frames (ORFs) of genomes have been a valuable starting point in the identification of virulence genes. For example, a comparison of the genomes of pathogenic *L. monocytogenes* and the closely related nonpathogenic *L. innocua* identified 270 genes that were specific to *L. monocytogenes* and 149 that were specific to *L. inocua* (Buchrieser et al. 2003). These genes were present in coding regions that were scattered over the genomes but typically different in guanosine plus cytosine (G+C) content than the 34% for Listeria-specific genes. The analysis has not so far identified new virulence genes. There are also exciting studies under way that exploit knowledge of the *E. coli* O157:H7 genome and the comparative virulence of other EHEC to identify genes that are associated with the extreme virulence of O157 EHEC compared with other Shiga toxin-producing *E. coli* (STEC), as well as variants within O157 in terms of host adaptation and virulence (M. A. Karmali, pers. comm.).

Genomic data, in combination with microarray technologies, have sometimes been used to probe not only bacterial metabolism in the host but also host changes in response to the presence of the bacteria. The enormous amount of data generated in these studies is often quite a challenge for analysis and interpretation. Typically, a large number of genes is up-regulated and down-regulated, and it is difficult to differentiate primary from secondary responses. The time at which a readout of mRNA is made is also critical as too long a delay may reveal only the steady state after much of the series of responses by bacteria and host have been completed. Like *in vivo* expression technology (IVET), these analyses identify genes expressed under certain conditions, and subsequent testing is needed to determine the subset of these genes that are essential for infection of the host and for disease. One meta-analysis of changes in gene expression by *Mycobacterium tuberculosis* in response to growth in macrophages showed poor concordance between the numerous studies (Kendall et al. 1994), suggesting that what may be apparently minor differences in experimental design, methodologies, and analysis

can have a dramatic effect on the results, which can essentially be useless.

Data mining of complete and incomplete genome sequences has been used to generate valuable information on virulence-related genes in bacteria. For example, 21 novel sequences that might encode ADP-ribosyl transferase activity were identified by this method in bacteria as diverse as *Streptococcus pyogenes*, *Mycobacterium avium*, *S.* Typhi, and *Mycoplasma pneumoniae* (Pallen et al. 2001). There is, however, a large gap between genomic analyses and functional genomics. This is exemplified by the fact that only about 60% of the genes of *E. coli* and 56% of the genes of *P. aeruginosa* have known functions. In addition, the presence of gene sequences does not necessarily mean that functional proteins are produced.

Newer rapid genome sequencing ability has permitted studies of metagenomics, thereby adding a new dimension to our capability to investigate pathogens in their natural environments. This will be particularly valuable in host niches such as the intestine, which have rich microbial communities whose interactions with pathogens are critical to health and disease.

EVOLUTION OF PATHOGENS—THE PATH TRAVELED MAY PROVIDE INSIGHTS INTO THE ROAD AHEAD

Evolutionary studies of pathogenic bacteria have shown that many have arisen by "quantum leaps" from nonpathogens as a result of acquisition of blocks of genetic material (that are sometimes very large) rather than by progressive mutations of existing genes. A large number of essential virulence genes are found on a variety of mobile genetic elements (bacteriophage, plasmids, transposons) that have been spread from other microbial sources through transformation, transduction, or conjugation, or combinations of these processes. The discovery of blocks of virulence genes on pathogenicity islands in phage insertion chromosomal hot spots has been one of the major surprises in recent years, reinforcing the concept that the evolution of virulence can be characterized in many cases as a dramatic process of "evolution by jerks" rather than as a slow, long-term, progressive refinement of "evolution by creeps" (point mutations, rearrangements) of existing genes to improve survival in different niches (although this is also important). Genomic islands, including pathogenicity islands, are dynamic and ancient integrative elements in the evolution of bacteria, including pathogens (Boyd et al. 2008). Clonal analysis of bacterial populations has been used to characterize the different times and populations at which these dramatic changes occurred.

For example, *Salmonella* evolved as a pathogen over the last 100 million years in three distinct phases and continues to evolve. Its infection by bacteriophages may have played a vital role in this process (Figueroa-Bossi et al. 2001). The first phase in this evolution involved acquisition of *Salmonella* pathogenicity island I (SPI 1). *Salmonella enterica* then diverged from *S. bongori* by acquisition of a second pathogenicity island (SPI 2). The final major phase was the process of branching into distinct phylogenetic groups, with a dramatic expansion of *S. enterica* subsp. I into warm-blooded animals (Bäumler et al. 1998). It may have thus evolved from a dinosaur into a mammalian pathogen. Some subsp. I serotypes further acquired the *Salmonella* virulence plasmid, which is characteristic of the major host-adapted serotypes, as well as the most virulent of the nonhost-adapted serotypes Enteritidis and Typhimurium. Possession of the virulence plasmid and its *spv* operon makes these serotypes particularly pathogenic (Bäumler et al. 2000).

The basis of the remarkably specific host adaptation of certain serotypes of *Salmonella* is unclear but may relate to the relative plasticity of the *Salmonella* genome afforded by phage-mediated transfer of a small number of host-specific virulence factors (Rabsch et al. 2002). It is in part a function of the presence of different types of specific fimbrial adhesins that recognize intestinal surfaces. For example, *S.* Typhimurium definitive phage type (DT) 49 and DT 104 appear to have a broad host range. However, in contrast, in Rock doves, *S.* Typhimurium var. Copenhagen is considered a specifically adapted subtype, with DT 2 and DT 99 being isolated almost exclusively from this species in Europe and North America (Rabsch et al. 2002). Certain strains of *S.* Typhimurium, particularly DT 40 and DT 160, may have become adapted to certain species of songbirds.

Not only has horizontal gene transfer through mobile genetic elements played a key role in the evolution of virulence, but many bacterial species are naturally competent for DNA molecules, so that DNA taken up from lysed bacteria within

microcolonies can lead to homologous recombination with mosaic genes that may give an advantage to their host. This may have both long-and short-term benefits to the organism. A classic example of immediate benefit is the formation of antigenically distinct variants of fimbrial adhesins by *Neisseria gonorrhoeae* selected out by the immune response of the host to the older major antigenic type.

In other cases, there has been an orderly buildup of virulence-related genes by horizontal transfer. This has been shown for *V. cholerae*, EHEC, and EPEC. In EPEC and EHEC, there has been clear evidence of selection for increasing virulence over time (Reid et al. 2000). The main advantages of the ability to induce diarrhea in the host are presumed to be an increase in the opportunity for acquisition of DNA in the intestine as colonization results in large numbers of pathogenic *E. coli* and enhanced transmission of bacteria in fluid stool. It is possible that there is coevolution to greater fitness, but this aspect has not been explored.

Bacteriophage encode many virulence, notably toxin, genes. Classic examples include botulinum toxin, cholera toxin, diphtheria toxin, Shiga toxin, and the superantigen genes of *S. aureus* and *S. pyogenes*. Phage may also transfer pathogenicity islands. The extensive recombination that is characteristic of bacteriophages may explain the variety of related toxin genes that they may encode. Plasmids may carry virulence genes that can be transferred through conjugation; in addition, plasmids commonly carry insertion sequences or transposons that can further mobilize virulence genes to the chromosome or to other plasmids. Plasmids, transposons, and integrons may carry antimicrobial resistance genes, some of which may be linked to virulence genes, suggesting that the use of antimicrobial drugs in animals may drive not only the evolution of resistance but possibly also the evolution of pathogens. The mechanisms of bacterial change are the same.

The emergence of pathogens has been associated not only with gain of genes, but also with their loss. Insertion sequences have had a dramatic impact on bacterial pathogen evolution, usually in the direction of reducing genome size and increasing host specificity. This is well illustrated in the evolution of *Bordetella pertussis* from the broad host range pathogen *B. bronchiseptica* to become an exclusively human pathogen (Preston et al. 2004).

The wide dissemination of families of virulence genes in unrelated bacterial populations may be explained by horizontal transfer. One example is the thiol-activated cholesterol binding cytolysins found particularly among gram-positive bacteria (e.g., listeriolysin, perfringolysin, pyolysin, streptolysin).

There is inherent competition between the ability of a bacterium to evolve through acquisition of virulence genes horizontally and fitness genes through mutation and rearrangement, and the need to maintain the integrity of the genome through the stabilizing mechanisms of DNA mutation repair, DNA restriction or modification, and other genetic barriers. As characterized by their diversity of hosts, their ability to cause quite diverse diseases, their ability to colonize different ecological niches, or their ability to acquire antimicrobial resistance genes, successful pathogens such as *E. coli* and *S. enterica* may be concluded to have an inherently greater ability to evolve, for example through a greater tendency to develop mutator mutants defective in DNA repair, than some perhaps more classical but certainly now unimportant pathogens such as *Erysipelothrix rhusiopathiae*. The low rate of evolution of some pathogens, such as *B. anthracis*, can be related to their lifestyle and environments.

Sometimes there are trade-offs between virulence and survival outside the host, as in the case of *Shigella* and the *cadA* gene. The *cadA* gene in each of the *Shigella* species has been independently inactivated (Day et al. 2001). *cadA* encodes lysine decarboxylase whose activity results in the production of cadaverine, which blocks the action of enterotoxins of *Shigella*, inhibits *Shigella*-induced polymorphonuclear leukocyte migration, and interferes with escape from the phagolysosome. This is considered a pathoadaptive mutation (a mutation that enhances survival by modification of traits that interfere with factors that are needed for survival in host tissues). The gene is retained by *E. coli* and is likely valuable in environments outside the host.

There are also instances in the evolution of pathogens where infection of animals may be regarded as almost accidental. For example, the ability of bacteria to survive and sometimes even thrive in the environment of macrophages as facultative intracellular pathogens may have first been developed by selection for their survival in amoebae; *Legionella pneumophila* is a classic example. Other pathogens, of which *P. aeruginosa* is the best example, likely evolved initially as plant pathogens but use the same virulence factors in causing disease in animals.

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2
Subversion of the Immune Response by Bacterial Pathogens

D. C. Hodgins and P. E. Shewen

INTRODUCTION

The wide variety of bacterial pathogens that successfully and continually infect humans and animal hosts, in spite of the broad capabilities of the immune system, is eloquent evidence that bacteria can avoid, block, defeat, inactivate, overcome, or otherwise subvert immune defenses. As understanding of the complexities of the immune system has advanced relentlessly, in parallel it has become clear that bacterial mechanisms of subversion operate at a high level of sophistication. Indeed, much of what is known about the immune system has been discovered by investigating successful pathogens.

This chapter summarizes current understanding of bacterial mechanisms that are considered subversive to immune responses, based on experimental evidence. Unfortunately, much of the evidence comes from *in vitro* studies using established cell lines and contrived model systems. These studies are useful in generating and testing hypotheses, but they have their limitations. *In vitro* studies cannot address whether the proposed mechanisms actually enhance bacterial replication, persistence, or transmission *in vivo* in the host species. Some bacteria appear to have multiple, overlapping mechanisms to achieve a single goal; the mechanism that is the most satisfying from an immuno-philosophical perspective may not be the most important quantitatively. In addition, subversive mechanisms operate in the context of an infected tissue, and in the face of changing immunological defenses. Bacterial mechanisms may need to be deployed at critical time points to be advantageous to the pathogen. Thus, *inhibition* of apoptosis of host cells may confer an advantage early in an infection (e.g., allowing an intracellular pathogen time to replicate), but *induction* of apoptosis may be advantageous at a later stage (e.g., to enhance dissemination of the pathogen). The material summarized in this chapter is presented in the context of existing evidence with the recognition that future work may show that some of the mechanisms do not have clinical relevance. Although the emphasis of this book is on bacterial pathogens of animals, some pathogens of humans have been included to illustrate mechanisms of subversion that have been well studied in the context of human medicine. Some of these mechanisms are doubtless also used by pathogens of animals and await discovery.

Immune Defenses

Immune defenses are conventionally classified as innate or adaptive. Innate defenses consist of cells and secreted substances that are not specific for particular pathogens and do not demonstrate immune memory (i.e., re-exposure to the same pathogen at a later date will lead to a response similar in magnitude and kinetics to that following initial exposure). Innate defenses include epithelial barriers to prevent bacterial penetration, clearance of mucus and trapped bacteria from the trachea by the action of ciliated epithelial cells, antimicrobial peptides, complement components, natural killer cells, and phagocytic cells such as neutrophils and macrophages. Adaptive defenses include immune responses that are clonally defined, for example, antibodies generated by antigen-specific B lymphocytes, and T lymphocytes specific for fragments of bacterial components. Adaptive responses require time to develop but typically are associated with immune memory (i.e., re-exposure to the same pathogen at a

later date will lead to a response of greater magnitude and accelerated kinetics compared with that following initial exposure). Adaptive responses cover a spectrum of activities, including antigen processing and presentation by antigen-presenting cells (APC), generation of antigen-specific effector and regulatory lymphocytes, and induction of memory cells. Bacteria of different species, serotypes, and strains may have diverse mechanisms to subvert the immune system at any of these points of defense and may intervene at multiple points simultaneously.

SUBVERSION OF INNATE IMMUNE RESPONSES

Evasion of Antimicrobial Peptides

Antimicrobial peptides comprise a large family of peptides with bactericidal activity against gram-positive and/or gram-negative organisms (Brogden et al. 2003). They are produced by neutrophils and macrophages, by epithelial cells of the skin, and by the respiratory, genitourinary, and gastrointestinal tracts. β-defensins are expressed by epithelial cells of the trachea following binding of lipopolysaccharides (LPS) to Toll-like receptor 4 (TLR4), with signalling via an NF-κB pathway. *In vitro* studies indicate that an unidentified virulence factor of *Bordetella bronchiseptica* delivered by a type III secretion system (TTSS) can sequester NF-κB in the cytoplasm of tracheal epithelial cells, reducing expression of β-defensins (Laube et al. 2006). In addition, staphylokinase produced by *Staphylococcus aureus* has been reported to bind β-defensins to form inactive complexes (Jin et al. 2004).

Impairment of Tracheal Clearance

The lower respiratory tract is exposed continually to microorganisms in inspired air. Some of these organisms become trapped in the mucus layer that covers the epithelial cells of the larger airways, and are transported to the epiglottis by the movement of cilia on the epithelial cells (mucociliary escalator). *Bordetella pertussis* secretes tracheal cytotoxin (TCT), which induces production of nitric oxide within epithelial cells, impairing their ability to clear mucus (Flak and Goldman 1996). *Pseudomonas aeruginosa* produces multiple toxins, including rhamnolipid, pyocyanin, and 1-hydroxyphenazine, which slow or stop ciliary beating (Read et al. 1992). Mycoplasmas of various species also induce derangements of cilial function (Young et al. 2000).

Adhesion and Penetration of Epithelial Barriers

Epithelial barriers constitute a very basic form of immune defense, and most species of pathogenic bacteria have mechanisms to breach these defenses. These mechanisms include recognized adhesins binding host cell receptors or host cell extracellular matrix (e.g., *Staphylococcus* spp.), fimbriae (e.g., enterotoxigenic *Escherichia coli*), type III secretion systems that inject bacterial proteins into host cells to act as receptors (e.g., enteropathogenic *E. coli*), or uptake by means of M cells overlying Peyer's patches in the intestine (e.g., *Salmonella enterica*). These mechanisms are described in detail in other chapters and will not be discussed further here.

Evasion of Complement-Mediated Opsonization, Chemotaxis, Killing, and Adjuvant Effects

The complement system consists of three overlapping enzyme cascades or pathways that contribute to innate immunity by: (1) targeting foreign material for enhanced uptake by phagocytic cells (opsonization), (2) generating pro-inflammatory molecules (anaphylotoxins), and (3) direct killing of pathogens by creation of membrane pores. The complement system also contributes to development of adaptive immunity (Nielsen and Leslie 2002) by: (1) targeting foreign material to APC, (2) augmenting antigen processing and presentation, (3) enhancing B cell activation, and (4) facilitating memory antibody responses. Bacterial processes reduce the efficiency of the complement system, therefore compromising immune defenses on multiple fronts.

The three complement pathways (classical, alternative, and lectin) vary in how their enzyme cascades are triggered, but are identical in their terminal components and final structures. All three pathways lead to the formation of C3 convertase complexes covalently linked to bacterial surfaces. These complexes cleave multiple units of plasma C3 to generate C3b fragments, which proceed to bind covalently to bacterial surfaces. Surface-bound C3b, associated with the C3 convertase, mediates cleavage of serum C5 to C5b, initiating assembly of a membrane-spanning pore (C5b-C6-C7-C8-C9, the "membrane attack complex" [MAC]). Insertion of sufficient of these pores in the membrane may lead to the death of the bacterium.

Table 2.1. Summary of Complement Pathways

Pathway	Initiating components	Components of the C3 convertase	Components of the C5 convertase	Regulating factors
Classical[a]	C1q, C1r, C1s, C4, C2	C4b2a[d]	C4b2a3b	CR1, C4BP
Alternative[b]	C3, factor B, factor D, properdin	C3bBb	$C3b_2Bb$	factor I, factor H
Lectin[c]	Mannose-binding lectin, ficolins, MASP-1,MASP-2, C4, C2	C4b2a	C4b2a3b	CR1, C4BP

[a]The classical pathway is initiated by binding of component C1q to complexes of antibodies with antigen, or by direct binding to some microbial structures. Sequential interaction of bound C1q with components C1r, C1s, C4, and C2 leads to formation of the C3 convertase.
[b]The alternative pathway is initiated when trace amounts of C3b formed by spontaneous breakdown of C3 bind to bacterial surfaces. Stabilization of C3b by serum factor B and cleavage by factor D leads to formation of the C3 convertase, which is stabilized by properdin.
[c]The lectin pathway is initiated by binding of mannose-binding lectin (MBL) or ficolins to bacterial sugars. Interaction of MASP-1 and MASP-2 with C4 and C2 leads to formation of the C3 convertase.
[d]The large fragment of C2 that associates with C4b is termed C2b by some authors.

Essential features of the three pathways are summarized in table 2.1 and fig. 2.1; standard textbooks of immunology (Murphy et al. 2008; Tizard 2009) can be consulted for more details.

Mechanisms of subversion of the complement system are summarized in table 2.2.

Inhibition of Activation of the Classical Complement Pathway

C1q is the first component in the classical pathway of complement activation. C1q can bind directly to some bacterial cell wall components, such as the lipoteichoic acid of gram-positive bacteria, or to C-reactive protein bound to bacterial phosphocholine, or to immune complexes consisting of IgM or IgG bound to antigen. M protein (fibrinogen-binding protein) of *Streptococcus equi*, protein A of *S. aureus*, and protein G and protein H of some species of streptococci bind the Fc region of IgG, preventing binding by C1q and blocking complement activation by the classical pathway (Peterson et al. 1977; Berge et al. 1997; Lewis et al. 2008). The strength of binding of IgG to these bacterial proteins varies with the sub-isotype of IgG; complement activation varies accordingly.

C1 esterase inhibitor (C1inh), a host complement regulatory protein, terminates the serine protease activity of C1r and C1s (classical complement pathway) and MASP-1 and MASP-2 (lectin pathway) by nonenzymatic reactions (Marr et al. 2007). C1inh also can inhibit formation of the C3 convertase of the alternative pathway (Jiang et al. 2001). *B. pertussis* can bind host C1inh to its surface, leading to resistance to serum-mediated killing *in vitro* (Marr et al. 2007). *E. coli* O157:H7 can secrete StcE, a protein that binds C1inh and tethers the resulting complex to membranes. C1inh bound in this way retains its ability to terminate complement activation, and increases resistance of *E. coli* to serum-mediated killing *in vitro* (Lathem et al. 2004).

C4b-binding protein (C4BP) is a host regulatory protein that degrades C4b bound to membranes and prevents further activation of the classical and lectin pathways of complement. Some strains of streptococci express Arp and Sir membrane proteins, members of the M protein family. These proteins have been shown to bind not only immunoglobulins but also C4BP. Bound C4BP retains its ability to degrade C4b (Thern et al. 1995). Berggard et al. (1997) have shown that *B. pertussis* can also bind C4BP. Barbosa et al. (2009) have demonstrated binding of C4BP to the surface of pathogenic but not nonpathogenic strains of *Leptospira*, and noted less insertion of the membrane-attack complex (MAC) into the membranes of the former following incubation in serum.

Inhibition of Activation of the Alternative Pathway

Sialic acids are common components of glycoconjugates on eukaryotic cell surfaces and play a role

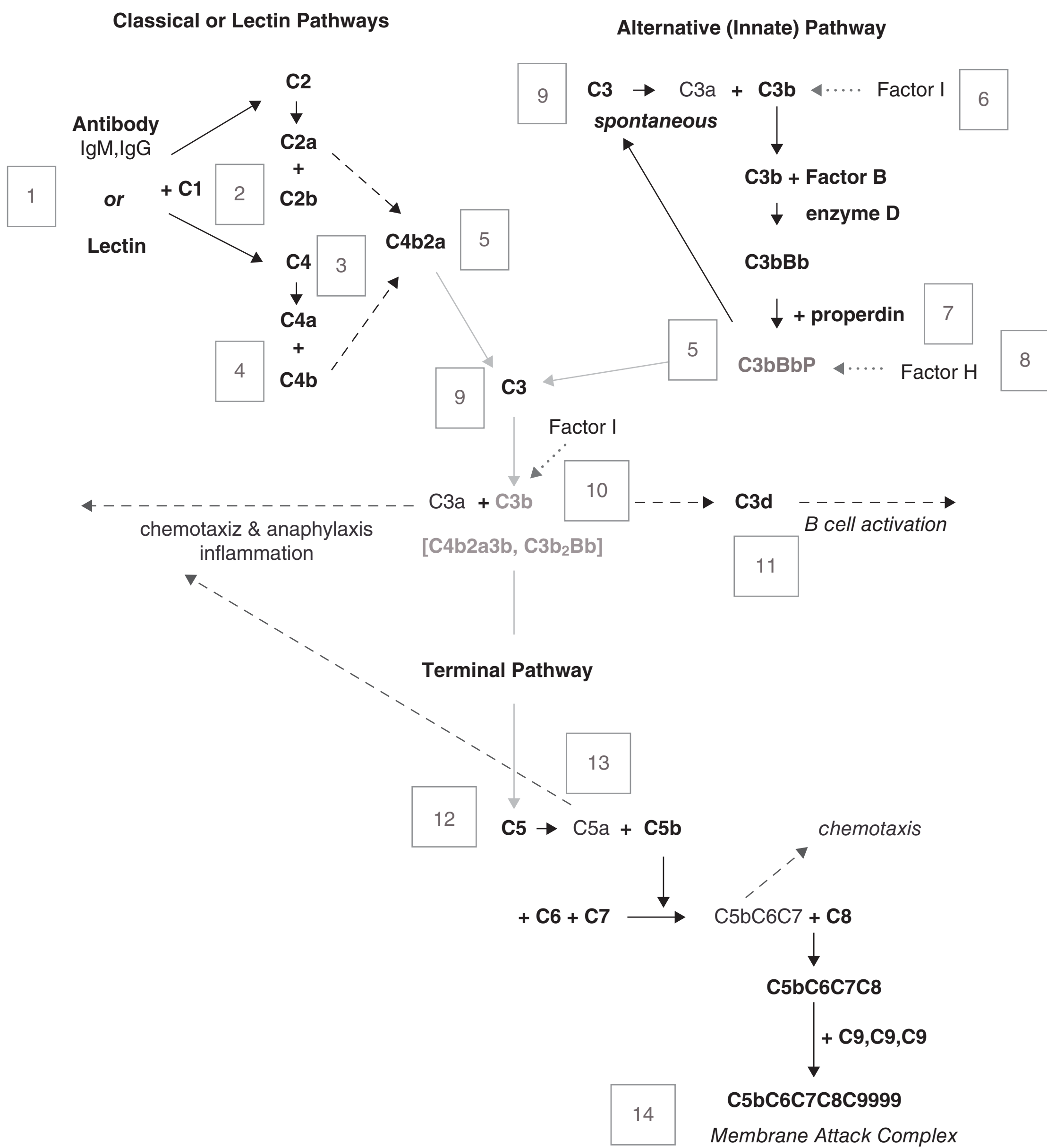

Figure 2.1. The complement cascade and microbial interference with complement activity. Numbers in boxes correspond to points vulnerable to microbial interference as indicated in table 2.2. C3 and C5 convertases are shown in blue. Factors in red are soluble split products associated with inflammation. Regulatory factors are shown in gray. (see color plate)

in blocking alternative pathway complement attack on host cells. Complement factor H binds to sialic acids and inactivates the C3 convertase complex (in association with factor I) by displacing and cleaving C3b. Some bacterial pathogens, such as *E. coli* K1, and *Neisseria meningitidis* can synthesize sialic acids, and generate polysialic acid capsules. Other pathogens, such as *Haemophilus*

Table 2.2. Examples of Bacterial Subversion of the Complement System

Mechanism (see fig. 2.1)	Example (numbers refer to boxes in fig.2.1)
Inhibition of the classical and lectin activation pathways	*Staphylococcus aureus* (1); Streptococci (1,4); *Escherichia coli* O157:H7 (2); *Bordetella pertussis* (2,3,4); *Leptospira* (4)
Inhibition of activation (alternative pathway)	*E. coli* K1, *Neisseria meningitidis*, Type III group B streptococci (6,8); *Borrelia burgdorfer*i, *Haemophilus influenzae*, *Histophilus somni*, *Pasteurella multocida*, streptococci (8)
Inhibition of C3 and C5 convertases	*S. aureus* (5,10)
Degradation of complement proteins	*Salmonella enterica* (4,10,12); *S. aureus* (1,6,10); *Streptococcus pyogenes* (1,6,7,8,9,10,13)
Blockage of C5a effects	*S. aureus* (13)
Blocking membrane attack complex (MAC)	*E. coli*, gram-positive bacteria in general, *Helicobacter pylori*, *P. multocida*, *Salmonella enterica* serovar Montevideo (14)
Effects on induction of adaptive immunity	*S. aureus* (11)

influenzae use transporter molecules to capture sialic acid released from host cells. Sialic acid can be incorporated into LPS by diverse species of bacteria including *Pasteurella multocida* and *Histophilus somni* (Severi et al. 2007).

Numerous pathogens have protein receptors for binding factor H or the closely related factor H-like protein 1 (FHL-1), thus limiting complement activation through the alternative pathway (reviewed extensively by Kraiczy and Würzner 2006; Lambris et al. 2008). Some of these pathogens (e.g., some strains of *Streptococcus pyogenes*) can also bind C4BP, thus having the capacity to limit complement activation by all three pathways. Some strains of *S pyogenes* bind factor H and/or FHL-1 by means of their M protein; some bind the same ligands by means of the surface protein fibronectin-binding protein (Fba; Pandiripally et al. 2002). *Borrelia burgdorferi* expresses up to five different complement regulator-acquiring surface proteins (CRASPs) that bind factor H and/or FHL-1 in active forms and enhance resistance to complement-mediated lysis (Hartmann et al. 2006).

Inhibition of C3 and C5 Convertases

C3 convertases and C5 convertases play crucial roles in amplification of complement activation, in generation of chemotactic and inflammatory anaphylatoxins, and in initiation of MAC. Five secreted proteins of *S. aureus* have been shown to have complement modulating properties. Extracellular fibrinogen-binding protein (Efb) and Efb homologous protein (Ehp) bind complement C3b and fragments thereof. There is a lack of consensus whether these proteins bind C3 in plasma and prevent its binding to bacterial surfaces by the classical and alternative pathways (Lee et al. 2004), or whether the primary effect of these proteins is to bind the alternative C3 convertase and the classical and alternative C5 convertases and prevent amplification of C3b production, and generation of C5a (anaphylatoxin, chemotactic for neutrophils) and C5b, the fragment responsible for the initiation of the MAC (Jongerius et al. 2007). Three related proteins of *S. aureus*, staphylococcal complement inhibitor (SCIN), SCIN-B, and SCIN-C bind to both classical and alternative C3 convertases, preventing further generation of C3b and downstream products (Jongerius et al. 2007).

Degradation of Complement Proteins

A surface-associated protease of *S. enterica*, PgtE, has been shown to cleave complement proteins C3b, C4b, and C5, and has been associated with resistance to killing by serum complement *in vitro*. PgtE also activates plasminogen to plasmin, further enhancing breakdown of C3b (Ramu et al. 2007). SpeB (a protease produced by 60% to 75% of *S. pyogenes* clinical isolates) cleaves a wide variety of host proteins, including extracellular matrix components, IgG, properdin, C3, and C3b (Chiang-Ni and Wu 2008). A C5a protease produced by the same

bacterium cleaves the C5a anaphylatoxin, reducing chemotactic stimulation and influx of neutrophils (Ji et al. 1996).

Inhibition of Inflammatory Effects of Anaphylatoxins

By-products of the complement cascades contribute to immune function. C3a, C4a, and C5a, small fragments generated by the cleavage of C3, C4, and C5, respectively, have pro-inflammatory effects. These "anaphylatoxins" are chemotactic for phagocytic cells and, by increasing local vascular permeability, also deliver soluble immune mediators to the site of infection. The most potent of these fragments is C5a. The majority of clinical isolates of *S. aureus* from humans produce chemotaxis inhibitory protein of *S. aureus* (CHIPS). This protein binds with high affinity to the human C5a receptor (C5aR) but with low affinity to two other related receptors, the second C5a receptor (C5L2) and formyl peptide receptor (FPR; Wright et al. 2007). Binding of CHIPS to C5aR thus can reduce the inflammatory effects of C5a, but the availability of other signalling pathways may limit the magnitude of this effect.

Inhibition of Membrane Damage by the Membrane Attack Complex

Bulky LPS and capsular polysaccharides create a barrier between surface bound C3 and C5 convertases and the (protected) bacterial membrane, which is the target for the MAC. Thus, bacterial strains characterized by smooth colony type (associated with LPS with long O chains) are more resistant to complement mediated lysis than strains with a rough colony type (LPS with incomplete O chains; Grossman et al. 1987). Similarly, bacterial strains that produce a polysaccharide capsule are more resistant than nonencapsulated strains to complement attack (Chung et al. 2001). In general, it is considered that gram-positive bacteria are resistant to lysis by MAC because their thick peptidoglycan layer protects the cell membrane (Moffitt and Frank 1994).

Protectin (CD59) is a host complement regulatory protein anchored in cell membranes by a glycophosphoinositol (GPI) tail. Protectin preserves host cell integrity by preventing assembly of the final C9 units into the MAC. Rautemaa et al. (2001) carried out an immunohistochemical study of biopsy material from the gastric mucosa of patients infected with *Helicobacter pylori*. They found that *H. pylori* in gastric pits had bound protectin; MAC were absent. In contrast, *H. pylori* on the surface epithelium lacked protectin, but did have MAC. Protectin has also been shown to bind to non-encapsulated, deep rough strains of *E. coli* (Rautemaa et al. 1998). Protectin with a GPI moiety (but not the soluble form of protectin) was incorporated into the bacterial membrane. Binding of protectin was associated with resistance to complement-mediated lysis. Rautemaa et al (1998) suggested that inflammatory reactions in tissue may lead to release of host protectin (with intact GPI) from cells, permitting uptake into bacterial membranes.

Inhibition of Effects of Complement on Adaptive Immunity

The discussion of effects on the complement system thus far has focused on innate functions. C3b, which has bound covalently to microbial surfaces, is cleaved eventually by host regulatory proteins (by factor H with factor I or by complement receptor 1 [CR1, also known as CD35]) to generate fragment C3d. C3d is not involved in enzymatic reactions to amplify complement reactions or in opsonization, but rather has a role in initiating B cell (adaptive, antibody) responses. Antigens, such as bacterial proteins, bound to C3d enhance B cell activation at low concentrations by binding complement receptor 2 (also known as CD21) and stimulating intracellular Ca^{2+} ion flux. Recently it has been demonstrated that Efb and Ehp of *S. aureus* not only bind to C3 and C5 convertases but also bind directly to C3d with high affinity and block interaction with CD21 (Ricklin et al. 2008). This has been shown to inhibit B cell Ca^{2+} ion flux in *vitro* and may serve to suppress adaptive antibody responses to *S. aureus*. CD21 is also expressed by some dendritic cells functioning as APC and by follicular dendritic cells involved in the generation of immune memory responses, but the effects of Efb and Ehp on these cells have not been determined.

Evasion of Phagocytosis

Microorganisms that penetrate into tissues are often internalized by specialized phagocytic cells. Macrophages resident in tissues, neutrophils infiltrating tissues in response to chemotactic signals, and blood monocytes (maturing into macrophages as they migrate) can function as phagocytic cells. Various pattern-recognition receptors (PRRs; e.g.,

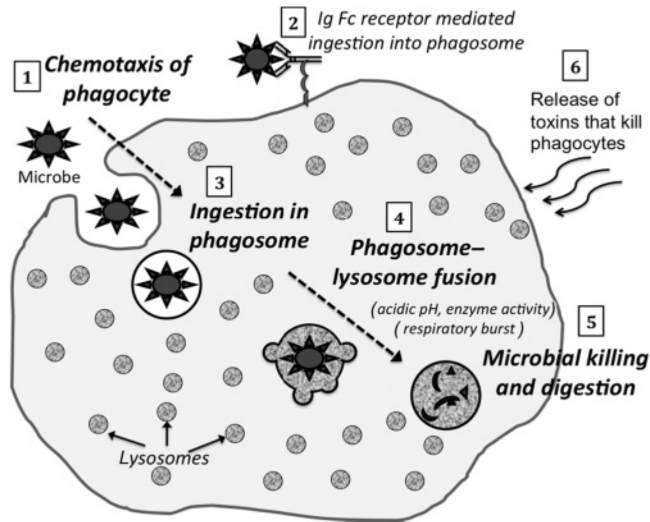

Figure 2.2. Microbial interference with phagocytic cell function. Numbers in boxes correspond to points vulnerable to microbial interference as indicated in table 2.3.

Toll-like receptors, macrophage mannose receptors, scavenger receptors, and FMet-Leu-Phe [fMLP] receptors) on the phagocytes may be used to identify pathogen-associated molecular patterns (PAMPs) of bacteria, leading to activation of the phagocytic cell. Adherence of antibodies and/or fragments of complement protein C3 to bacteria may enhance their uptake by phagocytes via receptors for bound antibodies (Fc receptors) and complement receptors (CR1, CR3, CR4), a process termed opsonization. Engulfed bacteria become enclosed within a vacuole termed the phagosome. Within minutes, cytoplasmic granules (lysosomes) of the phagocyte begin to fuse with the phagosome to create a phagolysosome. During this time the pH inside the vacuole decreases, toxic reactive substances are produced (nitric oxide, superoxide anion, and hydrogen peroxide) in an "oxidative burst" or "respiratory burst," and, with the assistance of antimicrobial peptides, nonpathogenic bacteria are killed. Pathogenic bacteria subvert this process in a multitude of ways (fig. 2.2; table 2.3) as summarized below.

Blocking of Chemotaxis

As mentioned previously, complement fragments C3a and C5a generated during complement activation act as chemoattractants for phagocytic cells. Bacterial processes that minimize complement activation by any of the pathways will diminish chemotaxis. Cleavage of C5a (*S. pyogenes*; Chiang-Ni and Wu 2008) or blockage of C5a receptor interaction (*S. aureus*; Wright et al. 2007) will have similar effects.

Pertussis toxin (PT) produced by *B. pertussis* has been shown to reduce synthesis of chemokines by alveolar macrophages in a mouse model (Andreasen and Carbonetti 2008). In addition PT has a direct suppressive effect on neutrophils, preventing membrane signaling in response to stimulation with chemokines, thus preventing influx of these cells into infected lung tissue.

Bacillus anthracis produces two soluble toxins, edema toxin and lethal toxin, which affect multiple cell types of the immune system by interfering with cell signaling pathways. Edema toxin increases cyclic adenosine monophosphate (cAMP) concentrations in the cytosol, and lethal toxin cleaves multiple isoforms of mitogen-activated protein kinase kinases (MAPKKs). Working in concert, the toxins block expression of pro-inflammatory cytokines, impede motility of neutrophils, and inhibit the oxidative burst in neutrophils (Baldari et al. 2006).

Bacteria of various *Yersinia* species use TTSS to deliver Yop proteins to phagocytic cells. Yop

Table 2.3. Examples of Bacterial Subversion of Phagocytosis

Mechanism (see figure 2.2)	Example (numbers refer to boxes in figure 2.2)
Reduce chemotaxis of neutrophils	*Bordetella pertussis* (1)
Reduce expression of proinflammatory cytokines	*Bacillus anthracis* (1)
Impairment of cell migration	*B. anthracis* (1); *Pseudomonas aeruginosa*, *Yersinia* spp. (1,2,3,4)
Kill phagocytic cells	*Histophilus somni*, *Mannheimia haemolytica*, *Staphylococcus aureus* (6)
Evasion of uptake	*Pasteurella multocida*, *Streptococcus suis* (3)
Interference with opsonization	*H. somni,* staphylococci, streptococci (2)
Biofilm formation	*Staphylococcus epidermidis*, *P. aeruginosa* (3)
Bacteria escape from phagosomes into cytoplasm, then penetrate directly from cell to cell	*Listeria monocytogenes*, Rickettsia (3)
Inhibition of formation of phagolysosome	*Brucella*, *Legionella pneumophila*, *Mycobacterium tuberculosis* (4)
Intracellular persistence	*M. tuberculosis* (5)
Inhibition of oxidative processes	*Erysipelothrix rhusiopathiae*, *H. somni*, *Mycoplasma bovis*, *S. aureus* (5)

proteins act to prevent actin fiber rearrangement, thus preventing cell migration as well as phagocytosis (Heesemann et al. 2006). Similarly, exoenzyme S and exoenzyme T of *P. aeruginosa* are delivered to the cytoplasm of phagocytic cells by a TTSS, also blocking phagocytic function by effects on actin (Barbieri and Sun 2004).

Cytotoxic Effects against Phagocytes

Mannheimia haemolytica produces a pore-forming leukotoxin of the RTX family of toxins, which is cytolytic for macrophages, neutrophils, and lymphocytes from ruminant species (Shewen and Wilkie 1982). *H. somni*, also a member of the *Pasteurellaceae*, has been shown to induce apoptosis in bovine neutrophils by a contact-mediated mechanism (Yang et al. 1998). Some isolates of *S. aureus* from humans produce a pore-forming leukocidin that causes lysis or apoptosis of neutrophils (Boyle-Vavra and Daum 2007). For all of these pathogens, killing of phagocytes clearly will impair the ability to eliminate the pathogens by phagocytosis; however, release of inflammatory mediators by dying neutrophils may have greater importance in the pathogenesis of the disease processes.

Anti-phagocytic Effects of Bacterial Capsules

As described in the context of evasion of complement activation, the presence of bacterial capsules is often associated with virulence. In some instances, polysaccharide capsules may interfere with complement-mediated opsonization, reducing the efficiency of phagocytosis. In other cases, bacterial capsules may enhance resistance to phagocytosis by mechanisms independent of complement (Boyce and Adler 2000). Segura et al. (1998) studied phagocytosis of *Streptococcus suis* by murine peritoneal macrophages, and macrophage cell lines under non-opsonic culture conditions. A parental encapsulated strain was highly resistant to phagocytosis, but an unencapsulated isogenic mutant was readily phagocytosed, indicating that at least for this particular strain, capsule had antiphagocytic properties independent of the effects of complement and antibodies.

Interference with Binding of Fc Receptors

Protein A of *S. aureus*, protein G of streptococci and an increasing number of bacterial Ig-binding proteins have been documented to bind to the Fc portion of immunoglobulins (Rooijakkers et al. 2005; Corbeil 2007; Nitsche-Schmitz et al. 2007). Protein A binding of Fc regions of IgG may prevent binding by Fc receptors on phagocytic cells, blocking uptake and killing (Foster 2005; Rooijakkers et al. 2005). Although this mechanism is intuitive and plausible, the validity of this interpretation is being questioned (Lewis et al. 2008) because

protein A and Fc receptors bind to different sites on the Fc region (Wines et al. 2000).

Receptors for IgA (Fc alphaRI, also known as CD89) are present on neutrophils, monocytes, macrophages, Kupffer cells, and eosinophils. These receptors bind IgA complexed with bacterial antigens, mediate phagocytosis, and induce an oxidative burst response. Sir22 and Arp4 of *S. pyogenes*, β protein of Group B streptococci (Pleass et al. 2001) and SSL7 protein of *S. aureus* (Wines et al. 2006) have been reported to bind the Fc region of IgA in the same location bound by Fc alphaRI. Thus, these bacterial proteins inhibit binding of IgA-complexed antigens to receptors on phagocytes, and prevent uptake and killing.

Formation of Biofilms

The formation of biofilms by bacteria affects their metabolic activities and physical–chemical characteristics. Cerca et al. (2006) have reported excessive production of extracellular bacterial antigens in biofilm matrix by *Staphylococcus epidermidis*. Although antibodies could penetrate the biofilm, phagocytosis and killing of bacteria were impaired. Jesaitis et al. (2003) have examined neutrophil responses to *P. aeruginosa* biofilms. Neutrophils made contact with the biofilm but underwent limited degranulation, accumulated minimal hydrogen peroxide, and had reduced motility.

Escape of Ingested Bacteria from the Phagosome

Several species of obligate or facultative intracellular pathogens have the ability to escape from phagosomes, before phagolysosomal fusion can take effect, into the cytoplasm where immune control mechanisms are minimal. Whitworth et al. (2005) have reported that *Rickettsia* can escape from the phagosome into the cytoplasm within 30–50 min after infection. Hemolysin C and phospholipase D are expressed during the process of escape and are necessary to induce breakdown of the phagosomal membrane. Once in the cytoplasm, the bacteria multiply. A rickettsial surface protein, RickA, activates host Arp2/3, inducing actin polymerization, forcing the bacterium through the host cell membrane and through the membrane of any adjoining cell, forming a membrane vacuole in the process (Walker 2007). This is followed by escape from the newly generated vacuole, and proliferation in the cytoplasm of the new host cell without direct exposure of the pathogen to the hostile extracellular environment. *Listeria monocytogenes* makes use of a pore-forming toxin, listeriolysin O, and phospholipase enzymes to escape the phagosome and spread from cell to cell in a similar fashion (Schnupf and Portnoy 2007).

Inhibition of Phagolysosomal Fusion

An impressive list of pathogens is capable of surviving and thriving indefinitely within macrophages. In recent years considerable research effort has been expended to clarify the mechanisms used by *Mycobacterium tuberculosis* to persist within phagosomes after uptake by macrophages. Phagocytic uptake of *M. tuberculosis* is enhanced by binding of complement components to the bacterial surface, followed by interaction with complement receptors CR1, CR3, and CR4 on macrophages. Phagosomes are formed at the time of phagocytosis, but the maturation of the phagosome toward the phagolysosome state is stalled at what has been described as the "four minute phagosome" state (Russell et al. 2005) because of its limited functional capabilities. Features of this state are a pH of about 6.4 (compared with a pH of 5.0 or less in unimpaired phagosomes) and a lack of hydrolytic enzymes due to a failure to acquire components of late endosomes. Various pathways have been suggested as mediating phagosomal maturation arrest. It has been proposed that mycobacterial cell wall lipids inhibit Ca^{2+} flux in the cytoplasm of the macrophage, blocking cell signaling that would trigger lysosomal fusion (Vergne et al. 2004; Kusner 2005). A number of studies have focused on the role of Rab5, a small guanosine triphosphate (GTP)-binding protein on phagosomes. Early endosomal autoantigen 1 (EEA1), a regulator of endosomal fusion, normally interacts with Rab5 but is excluded from phagosomes under the influence of mycobacterial products (Fratti et al. 2001). Other authors have focused on the interaction of mycobacterial cell wall components with macrophage lipid rafts as a critical event in blocking phagosomal maturation (Welin et al. 2008). It is unclear whether the above experimental findings all represent elements in a single pathway to prevent maturation of the phagosome, or whether several mechanisms function simultaneously to achieve the same goal.

Legionella pneumophila is another bacterial pathogen that can survive in macrophages by preventing phagolysosomal fusion. Research has focused on how *Legionella* modify the phagosome

by uptake of membrane-derived vacuoles originating from the host cell endoplasmic reticulum or Golgi apparatus. These vacuoles provide a continuing supply of nutrients and expand the volume of the phagosome to accommodate the replicating bacteria (Isberg et al. 2009). It is unclear what role, if any, this process of acquiring host membrane components plays in preventing fusion with the lysosome. Acquisition of membrane vacuoles is dependent on host proteins involved in membrane trafficking, including components of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. A total of 27 *defect in organelle trafficking/intracellular multiplication* (Dot/Icm) genes have been identified in *L. pneumophila* which have a role in manipulation of host membrane vacuoles, or enhance bacterial multiplication in the phagosome (Isberg et al. 2009). Many of the encoded proteins function in translocation of bacterial products across the host membrane and resemble components of type IV secretion systems (Cambronne and Roy 2007). Study of Dot/Icm protein interactions has revealed considerable redundancy in protein function. This redundancy complicates design and interpretation of genetic studies that aim to identify key genes in bacterial virulence. *L. pneumophila* may make use of host proteins involved in autophagy. Autophagy is a process in which host cell membranes enclose cytoplasmic materials for targeting to the lysosome (Shintani and Klionsky 2004); *L. pneumophila* may use elements of the host mechanism to acquire membrane material, but interrupt the process before lysosomal fusion occurs.

Brucella persist in non-professional phagocytes by preventing phagolysosomal fusion. There is morphological evidence for involvement of host autophagy elements in the process. Continuing interaction of a modified phagosome with the endoplasmic reticulum is reminiscent of mechanisms used by *Legionella*. Establishment and/or maintenance of a favourable phagosomal environment are dependent on the function of the VirB protein in a type IV secretion system (Celli et al. 2003).

Intracellular Persistence in Activated Macrophages

Mycobacterium tuberculosis survives within macrophages following phagocytosis, by inhibiting phagolysosomal fusion. However, survival is more difficult within macrophages that have been activated by IFN-γ produced by pathogen-specific T cells. Activated macrophages recover the ability to acidify phagosomes, and a pH of 4.5 can be maintained. Vandal et al. (2008) have recently identified a serine protease, Rv3671c, which permits *M. tuberculosis* to maintain an intrabacterial pH of more than 7.0 under these conditions, empowering the bacteria to survive even in activated macrophages.

Inhibition of the Oxidative Burst in Phagocytes

Bacteria that do not evade uptake by phagocytes and do not have mechanisms to prevent maturation of the phagosome must confront the ability of neutrophils and macrophages to generate reactive oxygen and nitrogen compounds. Some strains of some species of bacteria have enzymes at their disposal which can minimize the toxic effects of the hydrogen peroxide, nitric oxide, and related compounds generated by phagocytes. The practical issue is whether bacteria can express these enzymes soon enough and in large enough quantities to change the outcome of an encounter with a phagocytic cell. *S. aureus* produces two superoxide dismutases, sodA and sodM, in response to exposure to superoxide radicals (O_2^-). In a mouse challenge model, isogenic mutant strains of bacteria lacking one or both of these enzymes demonstrated reduced virulence (Karavolos et al. 2003). *S. aureus* also produce three methionine sulphoxide reductases, which may reduce oxidative damage to bacterial proteins (Singh and Moskovitz 2003).

Virulent strains of *H. somni* are able to inhibit production of superoxide radicals in both macrophages and neutrophils (Howard et al. 2004). This suppressive effect was not mediated by soluble bacterial products, but was dependent on contact of the phagocytes with live *H. somni*. Thomas et al. (1991) have reported that unopsonized live *Mycoplasma bovis* can adhere to bovine neutrophils without stimulating phagocytosis or an oxidative burst. *M. bovis* is also able to inhibit the killing of other bacteria ingested by neutrophils. Shimoji et al. (1996) have examined oxidative burst responses of mouse macrophages to *Erysipelothrix rhusiopathiae in vitro*. The macrophages could mount brisk oxidative responses to acapsular mutant strains and to the virulent parental strain in the presence of immune serum. In nonimmune serum, macrophages could invoke oxidative responses to the mutant strains but

were inhibited from an oxidative response by the parental strain.

SUBVERSION OF ADAPTIVE IMMUNITY

The adaptive immune system offers many opportunities to bacteria for subversion. Because adaptive immune responses are antigen specific, bacteria can evade immune effectors by changing the antigens that are expressed. Immune responses that are dependent on antigen processing can be inhibited by killing or modulating APC. Bacterial toxins can interfere with activation of lymphocytes to prevent clonal expansion. Alternatively, polyclonal stimulation of lymphocytes can inhibit pathogen-specific responses by consuming limited resources of energy and nutrients on irrelevant activities. Manipulation by bacteria of the cytokines produced by immune cells can steer immune responses away from pathways with bactericidal potential. Finally, destruction of soluble products or immune-mediated suppression of cellular mediators can prevent effective intervention against bacterial pathogens.

Antigenic Variation

Mycoplasmas are considered to have the smallest size and smallest genomes (600–2200 kb) of free-living cells. In spite of their limited genetic capacity, a number of *Mycoplasma* species have mechanisms for generation of antigenic diversity of their surface antigens. Remarkably, different genetic processes are used by different species of *Mycoplasma* to obtain this diversity. Mycoplasmas have the capacity to undergo phase variation (rapid shift from antigen expression to non-expression and return) and also the ability to change the nature (amino acid sequence and mass) of the antigens expressed (reviewed in detail by Razin et al. 1998). Le Grand et al. (1996) have documented antigenic variation in *M. bovis* surface lipoproteins in response to culture with antisera and monoclonal antibodies of different specificities. Responses to some sera consisted of termination of expression of the target lipoprotein, while in other cases expression shifted to similar lipoproteins of a different size, or to an antigenically distinct lipoprotein.

Apoptosis or Lysis of Lymphocytes

The leukotoxin of *M. haemolytica* A1 is a secreted pore-forming toxin of the RTX family, produced during logarithmic growth, which can kill leukocytes from ruminant species. This cytotoxic effect clearly can impair clearance of bacteria by neutrophils and reduce the population of lymphocytes available to generate an adaptive immune response. The leukotoxin can also impact immune responses at sublytic concentrations, however, by inducing apoptosis of bovine lymphocytes by means of a caspase-9 dependent pathway (Atapattu and Czuprynski 2005).

Bacillus anthracis can induce apoptosis not only of lymphoid cells, but also of macrophages and dendritic cells through the action of LT and ET toxins (Baldari et al. 2006).

Salmonella enterica serovar Typhimurium induces apoptosis in dendritic cells and macrophages. SipB, encoded in *Salmonella* pathogenicity island 1 (SPI-1), is delivered to the cytoplasm of the host cell by a TTSS where it activates host cell caspase-1, triggering apoptosis (Biedzka-Sarek and Skurnik 2006).

Inhibition of Lymphocyte Proliferation

The leukotoxin of *M. haemolytica* A1 can cause lysis of ruminant leukocytes, or apoptosis at lower concentrations. More subtle effects on lymphocyte function are also evident. Majury and Shewen (1991) have demonstrated a suppressive effect of the leukotoxin on proliferative responses of bovine lymphocytes to mitogens, and also on recall responses of lymphocytes from cattle vaccinated with Bacillus Calmette-Guérin.

Superantigens

T cell superantigens are protein toxins that can bind major histocompatibility complex (MHC) class II molecules and select T cell receptor (TCR) Vβ chains simultaneously (White et al. 1989). In the process, a considerable proportion of all T cells are stimulated, irrespective of their TCR specificity. Forty superantigens produced by *S. aureus* and *S. pyogenes* have been identified, as well as one produced by *Mycoplasma arthritidis* and three produced by *Yersinia pseudotuberculosis* (Fraser and Proft 2008). Early studies concluded that the trimolecular interaction of superantigen, MHC class II, and TCR Vβ was independent of the peptide bound in the MHC groove, but this has not been found to be consistent among all of the identified superantigens. Formation of the trimolecular complex is followed by a rapid release of multiple cytokines, especially IL-2, IFN-γ, and TNF-α, by the affected

T cells, leading to toxic shock syndrome in humans. In horses, superantigens produced by *S. equi* may contribute to the high fever, neutrophilia, and fibrinogenemia observed in strangles (Chapter 3). There is some doubt as to what benefit superantigens bestow on the bacteria which produce them. It has been suggested that early effects of the toxins on phagocytes may be more important than the later systemic effects of induced cytokines (Fraser and Proft 2008). Alternatively, stimulation of innumerable clones of T cells irrelevant to the infection may serve to impede the antigen-specific responses required to control the pathogen of interest.

In addition to superantigens that stimulate T cell subpopulations, bacterial products have also been identified that serve as B cell superantigens. In particular, *S. aureus* protein A can bind to the Fab region of human B cells expressing V_H3 family (framework) gene segments. This binding occurs by means of a region of protein A that is distinct from the region that binds the Fc portion of IgG. The site in the Fab region that is bound is distinct from the antigen-binding site (Silverman and Goodyear 2006). Studies conducted in mice suggest that cross-linking of Fab regions by protein A can lead to initial activation of B cells followed by apoptosis of these cells. This potentially may result in loss of the corresponding antigenic specificities. The clinical importance of B cell superantigens remains unclear, because experimental findings may differ depending to the size of bacterial inoculum used in mouse studies (Palmqvist et al. 2005; Silverman and Goodyear 2006).

Effects on Cytokine Expression

Immune responses require the involvement of many cell types; coordination and differential regulation of these cells are essential for control and elimination of the wide variety of pathogens that confront the immune system. Cytokines play an essential role in this process—activating some cells, suppressing others, inducing or inhibiting expression of membrane receptors, and regulating secretion of soluble mediators. The precise mixture of cytokines that is generated, their kinetics, and interactions can be decisive in defining the outcome of bacterial infections. Different immune responses are appropriate for control of extracellular and intracellular bacterial pathogens. In general terms, antibodies are most effective against extracellular bacteria, while cell-mediated responses (responses mediated by cytotoxic T cells and macrophage activating T cells) can be essential for control of intracellular bacteria. Subsets of CD4+ T helper (Th) lymphocytes have been classified based on the repertoire of cytokines produced by them. Th1 cells produce IFN-γ and IL-2 but minimal IL-4 or IL-10. Cytokines secreted by Th1 cells can activate macrophages to enhance killing of intracellular bacteria, and can also stimulate production of particular sub-isotypes of IgG antibodies by B cells. Th2 cells produce IL-4 and IL-10 but minimal IFN-γ or IL-2. Cytokines secreted by Th2 cells can stimulate production of particular sub-isotypes of IgG antibodies as well as production of IgE. Both Th cell types can produce various other cytokines besides the "signature cytokines." Additional Th classifications have been described (Th17, Treg) and new subsets will doubtless be identified. The Th1/Th2 paradigm was first proposed in the context of mice and has since been applied extensively in studies of disease pathogenesis in humans. How broadly the concept can be applied will become evident as more data accumulate from a wider representation of species.

Diverting an immune response dominated by Th1 cells (type 1 response) to an immune response dominated by Th2 cells (type 2 response) may be particularly advantageous for intracellular pathogens. In human infections with *Mycobacterium leprae*, two extremes of clinical disease have been described: tuberculoid leprosy and lepromatous leprosy. In the former, Th1 cells predominate in the skin lesions and few or no viable *M. leprae* can be identified in the lesions. In the latter, there is evidence of a type 2 response, and large numbers of viable organisms are present (Modlin 1994). It is of considerable scientific and practical interest whether the pathogen is capable of steering the immune response from a type 1 to a type 2 response, or whether this is controlled by genetic or environmental factors. In the 1980s phenolic glycolipid 1 was identified as a component of *M. leprae* with suppressive effects on lymphocyte proliferation for patients with lepromatous leprosy, but not tuberculoid leprosy (Mehra et al. 1984), but this did not address the issue of immune steering. Recent studies have focused on host genetics since over 90% of people exposed to *M. leprae* do not become symptomatic (Alter et al. 2008). Class II HLA genes and polymorphisms of the TNF-α promoter—vitamin D receptor, TLR2, and *PARK2* (a gene associated with early onset Parkinson's disease)—have been associated either

with susceptibility to clinical leprosy in general or with the tuberculoid versus lepromatous forms (Fitness et al. 2002; Schurr et al. 2006). It must not be overlooked that the dose of bacteria on initial exposure may also determine Th1/Th2 bias (Power et al. 1998). In spite of decades of research, it remains unclear whether *M. leprae* can steer the host's immune response from a type 1 response to type 2, or whether host or environmental factors are responsible.

Rhodococcus equi is an important cause of pneumonia in young foals (but not in adult horses), and can also induce pneumonia in immunocompromised humans. Like *M. leprae*, *R. equi* can survive in macrophages and protective immunity is considered dependent on a type 1 immune response (reviewed by Meijer and Prescott 2004). As with *M. leprae*, the question arises whether *R. equi* can steer the immune response away from a protective type 1 to an ineffective type 2 response. Giguère et al. (1999) examined T cell cytokine responses in foals challenged with virulent *R. equi* or with a plasmid-cured derivative (control foals). In foals receiving virulent *R. equi*, expression of IFN-γ was significantly lower in CD4+ T cells in bronchial lymph node (BLN), but IL-10 expression did not differ from that of control foals. In contrast, in lung tissue, expression of IFN-γ was similar in the two groups, but expression of IL-10 was significantly higher in the foals receiving virulent organisms. It is unclear which cytokine(s) in which tissue (or cell population) should be considered (most) representative of the host's immune response to *R. equi*. Jacks et al., (2007) investigated the hypothesis that limited IFN-γ responses in foals prevent adequate Th1 responses. Low challenge doses of *R. equi* were used (1×10^6 CFU per foal versus 2.5×10^9 CFU per foal in the study by Giguère et al. 1999) to better simulate natural exposure to the pathogen. Expression of IFN-γ by mononuclear cells from BLN stimulated *in vitro* with *R. equi* antigens was significantly higher for infected foals than for infected adults. Expression of IL-4 was significantly lower for infected foals. Neither of these findings supports the view that foals have an inherent inability to mount Th1 responses to *R. equi*. It remains unclear whether *R. equi* can steer the host's immune response from a type 1 response to type 2, or whether host (age?) or environmental (dose and route associated with natural exposure?) factors are responsible. These issues are discussed in detail in Chapter 9.

DC-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN, CD209) is a C-type lectin PRR expressed by dendritic cells in the dermis, lungs, lymph nodes, gastrointestinal tract, and lymph nodes (Van Kooyk and Geijtenbeek 2003). It functions in capture and uptake of microbes based on a high affinity for mannose-containing carbohydrates. *M tuberculosis* produces a cell wall containing a mannose capped form of lipoarabinomannan (ManLAM), which binds readily to DC-SIGN. ManLAM has been shown to inhibit activation of dendritic cells and to induce expression of IL-10, a cytokine that suppresses the type 1 T cell responses needed to control mycobacterial infections (Geijtenbeek et al. 2002). Thus, a cell wall component of *M. tuberculosis* plays an active role in manipulating cytokine expression, and in steering the immune system away from a protective response.

The effects of infection with *B. bronchiseptica* on functions of macrophages cells have been investigated in a mouse model by Siciliano et al. (2006). *B. bronchiseptica* produces an adenylate cyclase toxin that it can deliver to the cytoplasm of host cells by a TTSS. Macrophages infected in culture were inhibited in their ability to present ovalbumin peptide to transgenic CD4+ T cells expressing an ovalbumin-specific TCR. Mutant *B. bronchiseptica* lacking adenylate cyclase were used as controls. The authors showed that suppression of T cell proliferation was attributable to production of prostaglandin E_2 (PGE_2). Expression of IL-10, IL-23, and IL-17 were increased; expression of TNFα was decreased; and expression of IFN-γ and IL-12 was negligible. *B. bronchiseptica* was not only able to survive in macrophages, but also modified host cell function and the spectrum of cytokines that was produced.

Subversion of T Regulatory Cells

In recent years there has been intense interest in the role of the Treg subset of T lymphocytes (distinct from Th1 and Th2 populations) in chronic bacterial infections. This subset can suppress immune reactivity through production of IL-10 and/or transforming growth factor-β (TGF-β).

Tregs have been classified into naturally occurring and inducible subsets. Tregs can be CD4+ or CD8+ and may express CD25 and/or the forkhead transcription factor FoxP3 (reviewed by Joosten and Ottenhoff 2008). Adoptive transfer experiments in mice challenged with *M. tuberculosis* have

demonstrated a suppressive effect of Treg cells on clearance of intracellular bacteria. Rag1$^{-/-}$ mice (lacking B and T cells) received sorted T cells from naïve mice. The cell populations that were infused consisted of CD4+ T cells depleted of CD25+ cells (CD4+CD25– T cells) or CD4+CD25+ Treg cells. Rag1$^{-/-}$ mice receiving CD4+CD25– T cells cleared mycobacteria significantly better from lung and spleen than did wild-type immune competent mice. Rag1$^{-/-}$ mice that received CD4+CD25+ Treg cells in addition to CD4+CD25– T cells had significantly more viable bacteria in lung and spleen than those receiving only CD4+CD25– T cells (Kursar et al. 2007). Further studies are needed to define the mechanisms of suppression by Treg cells in this experimental model. It remains unclear whether mycobacteria can augment the numbers or activity of Treg in natural infections, or whether Treg activity is controlled by host or environmental factors.

Degradation of Immunoglobulins

A number of bacterial pathogens of humans, including *H. influenzae*, *Streptococcus pneumoniae*, and *Neisseria* species have been documented to produce proteases that cleave human IgA1 (Mistry and Stockley 2006). Cleavage occurs in the hinge region of IgA1, generating Fab and Fc fragments. It has been suggested that Fab fragments may bind bacterial antigens and inhibit binding of intact antibodies capable of mediating opsonization or phagocytosis. Efforts to document production of IgA proteases by pathogens of domestic animals have identified two *Ureaplasma* isolates from dogs that cleave canine IgA (Kapatais-Zoumbos et al. 1985; Proctor and Manning 1990).

A protease activity against bovine IgG1 has been reported in logarithmic phase culture supernatant of *M. haemolytica* A1 (Lee and Shewen 1996). The protease activity was present in a partially purified fraction that contained minimal leukotoxic activity but did contain a sialoglycoprotease. IgM and IgA were not cleaved.

CONCLUSION

As stated at the outset, the mechanisms by which pathogens evade or subvert host defenses are myriad and diverse. The ability of the host to control infection and prevent disease can be compromised at many levels encompassing both innate and acquired immune defenses. Many species of bacteria utilize multiple techniques of subversion, and indeed some individual bacterial proteins consist of a series of domains, each with an independent means of subversion. Combined, these mechanisms provide advantages with respect to bacterial replication, persistence, and transmission. They also provide clues for development of strategies with potential to counter bacterial virulence and in so doing assist the host. Thus, continued elucidation of the ways in which bacteria circumvent host immunity has the potential to reveal effective targets for vaccination or pharmaceutical interventions, some of which may have broad applicability across families of organisms that use similar subversive tactics.

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3
Evolution of Bacterial Virulence

P. Boerlin

WHAT ARE PATHOGENS AND HOW DO THEY EMERGE?

The definitions of virulence and pathogenicity and what makes the difference between a pathogenic and a nonpathogenic bacterium have been the subject of many controversial discussions. This is the case particularly because of the increasing importance of opportunistic infections in debilitated and immunodeficient patients, which blur the distinction between pathogens and commensals (Casadevall and Pirofski 2000). The most widely accepted definitions of pathogenicity and virulence are "the capacity of a microbe to cause damage in a host" and "the relative capacity of a microbe to cause damage in a host," respectively (Casadevall and Pirofski 1999). However, the capacity of a microorganism to cause damage is not a function of just the pathogen. It also depends on the host and on the circumstances of the encounter between the host and the pathogen (Casadevall and Pirofski 2003). To add to this complex situation, the ability of a pathogen to cause damage resulting in disease in a host is usually the consequence of interactions among several bacterial factors. Defining which factors are really responsible for causing damage to the host and are therefore virulence factors has also remained a controversial issue (Casadevall and Pirofski 1999 2001), resulting in the delineation of a variety of more or less distinct virulence factor categories (Wassenaar and Gaastra 2001).

The origins and circumstances leading to the emergence of most new pathogens out of anonymity are unclear and hypothetical. Among the many publications that describe and review the emergence of new pathogens, most are dedicated either to human pathogens or to rapidly evolving RNA viruses (Woolhouse et al. 2005). Only a few bacterial pathogens of animals have emerged in the recent past, and the related literature is very limited. However, researchers have developed general theoretical frameworks on the emergence of human pathogens that are of relevance and can be adapted for bacterial pathogens of animals (Woolhouse and Gaunt 2007).

Pathogenicity and virulence may be the result of a variety of encounters and adaptations between a host and a microorganism. Different levels of interaction or stages in the evolution of a microorganism toward a full blown infectious pathogen can be distinguished (Woolhouse and Gaunt 2007; Wolfe et al. 2007). The first stage (figure 3.1) in the emergence of a pathogen is the encounter with a new host or new body compartment of an already existing host. If the bacterium acquires a sufficiently broad repertoire of survival mechanisms, an infection with this "fortuitous" pathogen may take place (stage 2 in figure 3.1). Such a newly emerged "pathogen" may just be using tools that evolved to promote its survival in a completely different environment/host and circumstances, resulting *de facto* in virulence. *Legionella pneumophila* represents a good example of such a fortuitous pathogen, which is thought to use very complex transmission strategies and a variety of virulence factors that evolved for survival in protozoans but also cause damage in humans (Abu Kwaik et al. 1998; Albert-Weissenberger et al. 2007).

Repeated encounters between a fortuitous pathogen and a new host or penetration into a new body compartment may be frequent or intensive enough

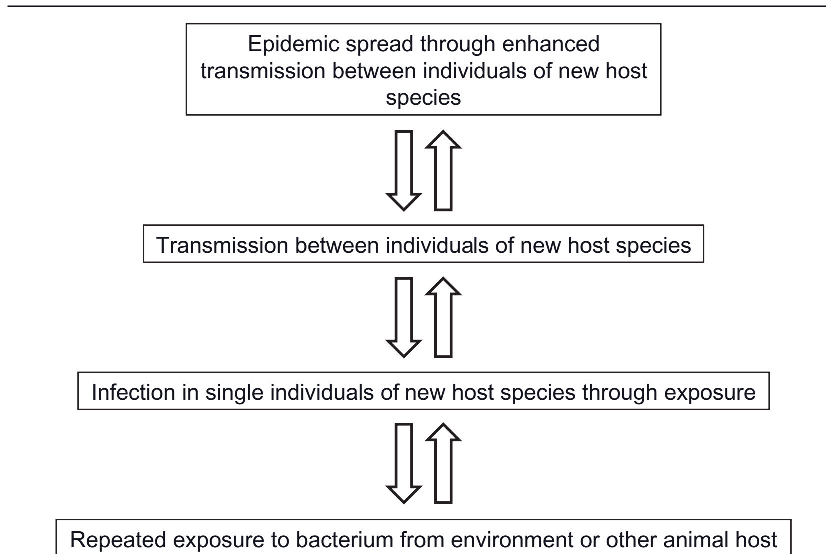

Figure 3.1. Theoretical frame for the emergence of new pathogens and evolution of virulence. Bacterial pathogens may stop their evolution at any step in this pathway, and, depending on the circumstances, they may even revert to a lower step. Changes from one step to the next may be triggered by the acquisition of new characteristics by a microorganism through mutations or HGT or by environmental changes in a broad sense. The latter include, for instance, increased exposure of the host by increased contact with another wild or domestic host species, penetration of new environments by hosts, increased exposure to vectors, increased host density or changes in natural or learned behavior facilitating transmission, changes in management practices and hygiene standards, changes in innate or acquired immune status (immunodeficient subpopulation), and genetic predisposition (inbreeding, introduction of new breeds or genetic lines).

to allow for the random emergence of a rare mutant with improved tools for survival and multiplication in this new environment. The interaction of a fortuitous pathogen with a new host is likely to represent in most cases a dead end for the microorganism. However, if it results in the survival and persistence of the microorganisms in some hosts, followed by return to its original environment or transmission to another susceptible host, then a new infectious pathogen is born with the capacity of being transmitted from one individual to another (level 3 in figure 3.1). Depending on the level of transmissibility, persistence, and exposure, this can lead to disease outbreaks of variable amplitude.

Finally, if the transmission level becomes high enough, the pathogen may cause epidemics and spread broadly across host populations (stage 4 in figure 3.1). It is thought that many emerging pathogens have followed this path of evolution. However, there is no necessity for a microorganism to follow this path to the end, and the majority of bacterial pathogens do not end up causing large epidemics. Both evolutionary changes leading to the acquisition of new characteristics in a pathogen (survival, transmissibility, invasion, and ability to cause damage) and changes in its environment or in its potential host population may trigger the move between levels of pathogenicity. The former will be the main subject of this chapter. The latter includes increased contact between potential pathogens and new hosts through ecological changes, changes in vector populations (climate change, hygiene), and changes in host populations (emergence of immunodeficient populations, increased stress and host density, changes in behavior promoting transmission, and genetic changes affecting susceptibility).

Tick-borne diseases and Lyme disease, in particular, are well-known examples of emergence of old

pathogens caused by ecological changes (Paddock and Yabsley 2007). In this particular case, changes in the environment and in white-tailed deer populations brought about by human activities led to repeated outbreaks and an increase in endemic rates of Lyme disease in north-eastern North America (Steere et al. 2004). It does not take much imagination to think of many similar situations in domestic animal populations and to understand the potential for the emergence of new pathogens in both companion and farm animals.

BACTERIAL FITNESS AND VIRULENCE

The model of emergence of new pathogens described earlier should not be understood as a one-way evolution. Pathogenicity is just one among numerous strategies available to a microorganism to maintain its fitness and to remain competitive in the fight for limited resources in a frequently hostile environment. The evolution of pathogens does not always lead to a continuous increase in or maintenance of virulence. The deleterious effects of exploitation of the host may ultimately also affect the microorganism itself. Thus, pathogens have to evolve ways to take advantage of their host without compromising their own survival and multiplication (i.e., to maintain a high fitness level in terms of population biology). Common wisdom would suggest that the general evolutionary trend of pathogenic microorganisms would be toward commensalism. High virulence levels could be a sign of recent colonization of a new host by a microbial species. The longer a microorganism persists and associates with a host species, the less virulent it would become until it reaches the climax of commensalism.

Unfortunately, this rather simple reasoning does not fit with the natural history of many pathogens. A positive relationship between virulence levels and transmissibility of microorganisms has been postulated in order to accommodate the inconsistencies of the common wisdom theory. Experimental and observational studies on some important pathogens showing that highly virulent microorganisms are often readily transmissible whereas less virulent ones are less easily or less frequently transmitted tend to support this hypothesis, known as Ewald's hypothesis (Ewald 1993). Some recent studies on bacterial pathogens (Wickham et al. 2007; Marquina-Castillo et al. 2009) continue to provide support for this hypothesis, also known as the trade-off hypothesis (Anderson and May 1982). However, the reality of infectious disease is still more complex, and this simple theory is not able to explain many cases of virulence and transmission patterns observed in the field (Levin 1996; Ebert and Bull 2003). Many researchers have questioned its validity, but it is likely that including more case-specific variables into each of the diverse models of virulence evolution would reconcile the trade-off hypothesis with the diverse biological realities seen in the field (Alizon et al. 2009).

The influence of host variability and host population structure (Alizon et al. 2009) on the evolution of virulence also remains to be explored. This variability is very broad and extends from the within individual host variability (consider the numerous potential anatomic locations of a pathogen within a host) to the between-host variability, which may even encompass the variability between host species for multihost pathogens (Pfennig 2001; Woolhouse et al. 2001). The mechanisms governing the evolution of a microorganism toward a specialized or toward a generalized multihost pathogen are complex. The adaptation to a new host may, in some cases, encompass a trade-off between the development of virulence in a new host and the loss of virulence and fitness in a previous host (Ebert 1998), thus leading to the emergence of new specialized clones and, ultimately, of new host-adapted species (Pfennig 2001). Alternatively, pathogens may develop such a constant strong genetic polymorphism that individuals adapted to a new host and new situations will be present in each population, thus broadening the host range of the pathogen (Pfennig 2001; Woolhouse et al. 2001).

A pathogen may also evolve many different virulence mechanisms and express them at the required level only under certain conditions, depending on its actual host or environment (a phenomenon called polyphenism in evolution biology). Thus, not only the virulence factors of a pathogen *per se* but also their regulation is subject to evolutionary constraints (Pfennig 2001). Competition between microorganisms for a host or an ecological niche within the host is another important factor driving the evolution of virulence of some pathogens. Finally, the influence of coevolution of a host and pathogen remains an as yet barely studied topic beyond mathematical and simplified experimental models. Nevertheless, examples of field studies on coevolution are emerging both at the population level (Maillard et al.

2008) and at the molecular level (Stavrinides et al. 2008; Shames et al. 2009).

In light of the preceding comments, it should become clear that the complexity of the mechanisms governing the evolution of virulence is certain to overcome the simplicity of the actual theoretical models (Ebert and Bull 2003). Numerous discoveries have to be expected in this field before we can seriously envisage the development of strategies to manipulate the evolution of virulence (Ewald et al. 1998; Ebert and Bull 2003).

SOURCES OF GENETIC DIVERSITY, POPULATION STRUCTURE, AND GENOME PLASTICITY

Three major ways of evolution are available to bacteria in order to maintain variability and adaptability to new environments (Morschhauser et al. 2000): local sequence change through mutations, DNA rearrangements, and DNA acquisition (horizontal gene transfer [HGT]). Although this is not an absolute rule, mutations and internal rearrangements are thought to result mainly in stepwise adaptations and to be mostly involved in microevolution and in fine-tuning (genetic drift). On the contrary, HGT has frequently been associated with more drastic changes and in major macroevolutionary steps in the lifestyle and virulence of pathogens (genetic shifts).

Mutation rates are not always constant in the life of a bacterial population (Giraud et al. 2001). In the presence of environmental changes or other stresses, a diversity of mechanisms, inducible (for instance, the SOS system of bacteria) or temporarily fixed genetically in a population (mutator strains defective in the control of DNA replication fidelity (Rainey 1999)) may increase the mutation rates (Sundin and Weigand 2007). Although this phenomenon has been suggested to play a role in the adaptation of pathogens to the stressful host environment and in the evolution of virulence, only a few publications really support this hypothesis by practical examples other than pulmonary infections with *Pseudomonas aeruginosa* (Oliver et al. 2000; Hogardt et al. 2007), urinary tract infections with *Escherichia coli* (Denamur et al. 2002; Labat et al. 2005), and host adaptation of *Salmonella* (Nilsson et al. 2004).

Rearrangements, including the formation of deletions nicknamed "black holes" in the genome of pathogens, represent an important mechanism in the adaptation of pathogens to new hosts (Maurelli 2007) and will be discussed later in the section on genome reduction and decay.

Bacterial species vary greatly in their ability to accept foreign DNA and to integrate it into their own genome. A broad diversity of genetic behaviors can be observed in natural populations of bacterial pathogens, ranging from completely clonal (species in which HGT is not able to disrupt significantly the overall tree-like evolutionary structure resulting from the binary cell division of bacteria) to panmictic populations (i.e., populations with frequent exchange and rearrangement of genetic determinants resulting in an overall network-like evolutionary structure) reminiscent of eukaryote populations with a sexual reproduction cycle (Spratt and Maiden 1999; Feil and Spratt 2001). Despite this apparent range of evolutionary behaviors, advances in population genetics due to use of molecular biology tools, such as comparative genomics, show that bacteria rely mainly on HGT for the major steps in their evolution (Fitzgerald and Musser 2001; Pallen and Wren 2007; Touchon et al. 2009), in particular, with regard to the evolution of virulence (Ziebuhr et al. 1999; Ochman et al. 2000).

HGT is mediated by three major transfer mechanisms (see Syvanen and Kado 2002): transformation (direct uptake of naked DNA by competent bacteria), transduction (DNA transfer and recombination through bacteriophages), and conjugation (transfer of plasmid and/or chromosomal DNA through mating between a donor and a recipient bacterium). Examples of all three transfer mechanisms are known to occur for virulence determinants. Although HGT represents a very important first step in the evolution and adaptation of a pathogen to the adverse conditions encountered in their host, it is not the end of the road. After transfer, the foreign DNA has, first, to be stabilized and replicated efficiently and, second, to be expressed adequately. Finally, the newly acquired characteristics have to be fixed in the new bacterial host population, and the genetic background of the new host may play an important role in these later steps (Escobar-Paramo et al. 2004).

In the case of transformation, the first step of stabilization is predicted to occur through homologous recombination rather than through illegitimate incorporation, thus usually limiting the range of novelty introduced into the recipient to genes from closely related bacteria. Through their internally

encoded replication and integration functions, phages, plasmids, conjugative tranposons, and integrative conjugative elements (Burrus and Waldor 2004) seem to be able to transfer and stabilize new genes from more distant donors and to play a more important role in the evolution of virulence. Because of the selective advantage of coordinated regulation, genes transferred in clusters or as part of whole operons have more chances than single genes to be fixed in a new host (Lawrence 1999). Mutations, rearrangements, and recombinations within the genome of the new host are subsequently responsible for fine-tuning the expression of the newly acquired virulence characteristics (Ziebuhr et al. 1999).

Recent comparative genomic investigations confirm that HGT and stabilization of transferred genetic material favor the emergence of clusters of genes. They also suggest that hot spots are present within genomes, where the newly acquired genes tend to accumulate more frequently, thus not disrupting the structure and functionality of the core genome common to most members of a species (Touchon et al. 2009).

PATHOGENICITY ISLANDS

One of the major concepts that emerged during the past decade with regard to clustering of horizontally transferred genes is that of genomic islands (Hacker and Carniel 2001; Dobrindt et al. 2004). The term "island" was first used for clusters of virulence genes from uropathogenic *E. coli* called pathogenicity islands (PAIs; Hacker et al. 1990). They were later shown to be a widespread phenomenon in bacterial pathogens in general (Gal-Mor and Finlay 2006). PAIs, which represent a specific subgroup of genomic islands, encode a variety of virulence factors, such as toxins, superantigens, adhesins, invasins, iron uptake systems, type III and type IV secretion systems, and many other effectors that modulate the behavior of host cells. They are found in a large variety of animal pathogens including *E. coli*, *Salmonella enterica*, *Yersinia* spp., *Dichelobacter nodosus*, *Bacteroides fragilis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Listeria ivanovii*, and *Clostridium difficile* (Hacker and Kaper 2000). Except for their specific presence in pathogens and the fact that they encode virulence factors, PAIs share some general characteristics with other genomic islands (Hacker and Kaper 2000), which are listed in table 3.1.

A number of approaches have been developed and used to detect genomic islands either by laboratory experimentation or increasingly by *in silico* analysis of bacterial genomic sequences (Langille et al. 2008). These methods include subtractive hybridization to identify pathogen-specific sequences; screening regions flanking tRNA genes, regions with atypical G + C content or codon usage, genes associated with genetic element mobility and HGT; and island probing through detection of unstable regions of the genome (Dobrindt et al. 2004;

Table 3.1. Common Characteristics of PAIs

Characteristic	Origin or putative function
G + C content different from the core genomic DNA	Marker of foreign origin through horizontal transfer
Mosaic structure	Remnants of past recombinations and sign of successive adaptation to host(s)
Association with tRNA genes	Hot spots for integration and coregulation
Frequent location on chromosome	Stabilization
Relatively large size (10–200 kb)	Coordination of expression
Encoding one or several virulence factors	Adaptation to host(s)
Usually only in pathogenic species or clones	Adaptation to host(s)
Direct repeats at both ends	Remnants of integration events, maintenance of plasticity and mobility
Cryptic or functional mobility factor genes or parts of them	Remnants of integration events, maintenance of plasticity and mobility
Often unstable or mobile	Adaptability to new environments and hosts

Gal-Mor and Finlay 2006). Criteria such as G + C content and codon usage may lack sensitivity because differences between acquired and core genetic material tend to decrease over time and to disappear in very old PAIs.

The mechanisms of horizontal transfer of PAIs are still not well understood and may be multiple. The presence of some bacteriophage integrase genes or their remnants in PAIs and their propensity (similar to that of phages) to be inserted in the direct proximity of tRNA genes suggest that PAI transfer may be related to phage transduction. However, although examples relating HGT of genomic islands to bacteriophages, plasmids, conjugative transposons, and integrative conjugative elements have been described, recent comparative studies of integrase genes suggest that a majority of genomic islands may indeed represent independent mobile genetic elements unrelated to phages and other classical mobile elements (Boyd et al. 2009). Analysis of the evolutionary path of some PAIs and their flanking regions also suggests that in addition to the initial steps in mobility provided by site-specific recombination, homologous recombination may also play an important role in the dissemination of genomic islands within related host organisms (Schubert et al. 2009).

Different levels of stability have been observed among PAIs. Some PAIs are mobile and relatively plastic, whereas others have only nonfunctional remnants of mobility genes and seem to be subject to fewer recombination events. Integrase genes, transposase genes, and insertion sequences within PAIs as well as direct repeats at their ends are thought to allow PAIs in the early stages of their evolution to adapt their structures and functions to the needs of their new bacterial hosts or to the variable environment encountered during their life cycle. This is supported by the mosaic structures often evident in PAIs, showing that they undergo frequent and multiple recombination events. Some authors have also suggested that PAIs may stabilize over time in parallel to the adaptation of their functions and of their regulation networks to their host (Hacker and Kaper 2000). As for many other horizontally transferred elements, the foreign origin of PAIs is clear, but their origins have usually not been clearly elucidated.

Pathogens have found many ways of optimizing the expression of virulence genes from PAIs and to integrate them into more complex and global virulence regulatory networks (a phenomenon called pathoadaptation). In the simplest case, regulatory genes responsible for the expression of virulence factors of a PAI may be located directly on the island. This is, for instance, the case for the *hilD* and *hilA* genes of the *Salmonella* PAI-1 (Lostroh and Lee 2001). However, regulatory genes for virulence genes of PAIs may also be found at other locations. Conversely, regulatory genes from PAIs may regulate the expression of virulence genes located at other chromosomal locations and on plasmids (Saitoh et al. 2008). Cross talk between different PAIs has been observed, with regulatory genes for one PAI located on another PAI probably acquired at a different time in the evolution of the pathogen (Ahmer et al. 1999).

BACTERIOPHAGES AND THEIR ROLE IN PATHOGEN EVOLUTION AND VIRULENCE

Bacteriophages have long been known to play a role as carriers of toxin genes in important pathogens. Examples include the diphtheria toxin gene in *Corynebacterium diphteriae* and the pyrogenic toxin genes in group A streptococci. A growing number of other important examples of virulence genes transferred by bacteriophages have emerged in recent years (for a review, see, for instance, Brussow et al. 2004).

Depending on bacteriophage, host cell, and environmental conditions, bacteriophages can go through either a lytic cycle (in which they replicate within a cell and generate free viral particles that are able to infect other host cells) or a lysogenic cycle (in which they integrate and stay in the genome of their host as a prophage). A lytic cycle can be the basis for HGT through generalized transduction, but this is a relatively inefficient process for transfer. The alternation of lytic and lysogenic cycles resulting in either generalized transduction, specialized transduction, or lysogenic conversion seems a much more efficient way of transferring genetic material between bacterial cells.

In the case of generalized transduction, bacterial DNA from the infected cell is mistakenly packaged into a bacteriophage head and transferred into a new host cell, where it can possibly recombine or (more frequently) gets degraded. In the prophage state, imprecise excision of the bacteriophage DNA resulting in co-packaging of host DNA adjacent to the viral DNA can be responsible for transfer of

bacterial host DNA, resulting in specialized transduction. Thus, in both types of transductions, any kind of bacterial DNA can potentially be transferred horizontally. This is different from lysogenic conversion, in which specific genes (called morons) not related to functions essential for the bacteriophage replication are transferred and integrated into the bacterial genome upon lysogenization by a temperate phage.

Genome sequencing has demonstrated that in some species, prophages and remnants of bacteriophages form up to 20% of the bacterial genome (Casjens 2003). In some other species, bacteriophages may be only minor participants in HGT, and prophage sequences seem to be absent from their genomes (Brussow et al. 2004). However, the accumulation of multiple genome sequences from a broad diversity of strains demonstrates the variability of prophage content within each bacterial species rather than their complete absence in some hosts. Comparative genomics has also shown that the presence of several prophages with homologous sequences in a genome can be (as any other repeated sequence) a source of homologous recombination, resulting sometimes in deletion and inversion of significant parts of the genome. These recombinations also play an important role in phage diversification and in the generation of their typical modular or mosaic-like genetic organization (Casjens 2003).

Once a bacteriophage becomes integrated into the host chromosome, much of the selective pressure on its replication systems is relieved and, consequently, mutations and deletions in genes involved in such functions are prone to accumulate, leading to defective prophages. Only functions such as those of morons or phage functions of relevance for the host bacterium may ultimately be retained in decaying prophages (Brussow et al. 2004). However, in some cases, there may be an evolutionary advantage for both the bacteriophage and the host in the retention of at least some ability to switch back to a lytic cycle with spread of the phage to other hosts. This is thought to be the case for bacteriophages encoding Shiga toxins (Brabban et al. 2005). In this case, release of infectious phage particles that encode the production of Shiga toxin by a few Shiga-toxin-producing *E. coli* (STEC) may result in the massive infection of bystanders. This may lead to a substantial increase in Shiga-toxin production without the need for the lysis and sacrifice of many members of the original STEC population (Gamage et al. 2003).

As noted earlier, insertion of new genetic material into the genome of an organism is only a part of the process leading to changes in the virulence or emergence of a pathogen. In the vast majority of cases, the newly acquired virulence genes have to be integrated into preexisting regulatory and functional networks of nonphage genes. The sopEΦ prophage found in some *S. enterica* strains is a fascinating example of such an integration (Ehrbar and Hardt 2005). The expression of its moron, *sopE*, is under the control of regulatory genes from the *Salmonella* PAI-1 (SPI-1) and not of a regulator encoded by the prophage itself. In addition, the SopE effector protein needs a chaperone and a type III secretion system (TTSS) encoded by SPI-1 to be efficiently secreted and injected into host cells. Thus, neither the expression nor the components needed for the transport of *sopE* are encoded by sopEΦ, and one can only wonder at the circumstances that have brought together these unrelated elements into such a coordinated network.

These examples provide some insights into the important role that bacteriophages play in the evolution of bacterial pathogens, including not only the transfer of virulence factors but also the modification of bacterial surface antigens (Kropinski et al. 2007), and in the plasticity of the bacterial genome structure. More examples of the involvement of bacteriophages in the adaptation of pathogens and in their evolution can be found in recent reviews on this topic (e.g., Brussow et al. 2004), and many more are certain to be discovered with the massive number of bacterial genomes and bacteriophage sequences that are being generated.

ILLUSTRATIONS OF VIRULENCE EVOLUTION

Clonal and Parallel Evolution of Virulence in *Salmonella* and *Escherichia coli*

The genera *Escherichia* and *Salmonella* are closely related and share a common ancestor, which is presumed to have been a commensal bacterium adapted to the intestinal environment of animals. After divergence from *Escherichia*, some hundred million years ago, *Salmonella* began sequentially accumulating virulence characteristics through HGT. Up to one quarter of the *S. enterica* genome may have been acquired through HGT after this first speciation step (Porwollik and McClelland 2003), and PAIs seem to have played a central role in this

evolution (Hensel 2004). The ancestral *Salmonella* lineage seems to have first acquired the ability to adhere to the intestinal wall, to invade its epithelial cells, and to increase fluid secretion through the stepwise horizontal acquisition of diverse virulence genes (for instance, through the acquisition of SPI-1 and of other invasin and adhesin genes). These genes are found in practically all *Salmonella* and were acquired before the species *S. enterica* and *Salmonella bongori* diverged (Baumler 1997; Groisman and Ochman 1997; Porwollik and McClelland 2003).

Another PAI (SPI-5) was acquired during the same period (Mirold et al. 2001), as did possibly a third one (SPI-3) or at least some of its constituents (Blanc-Potard et al. 1999). The *S. enterica* lineage has subsequently acquired additional virulence factors allowing for the development of systemic infections, which are missing in *S. bongori*. This type of infection frequently leads to long-term shedding, thus compensating for the associated increase in death rate and allowing *S. enterica* to colonize a new ecological niche. The acquisition of the PAI SPI-2 with its factors involved in survival within macrophages seems to have been a major step in this latter direction (Baumler 1997; Groisman and Ochman 1997).

Salmonella enterica subsequently diversified into seven subspecies, six of which evolved toward commensalisms in poikilothermic animals, whereas the seventh (subspecies I) evolved as a pathogen of homeothermic animals. During the later steps of the evolution, the genes necessary for flagella phase switch were acquired, thus converting some *Salmonella* lineages, such as subspecies I, into diphasic organisms and leaving others in their monophasic state. *S. enterica* subspecies I further diversified into numerous serovars, some of which have developed an increased propensity to cause systemic infections in specific host species. This host adaptation is multifactorial (Eswarappa et al. 2008) and again associated with repeated HGT and recombinations (Baumler et al. 1998).

Although the events described earlier give a mostly linear, unidirectional, and clonal depiction, the evolution of virulence in *Salmonella* is punctuated by multiple back and forth changes with repeated acquisition, recombination, and deletion events. This phenomenon, already mentioned earlier in relation to bacteriophages, results in the mosaic structure of PAIs and in inconsistencies between the overall evolution of these elements and of some of their components. This is, for instance, the case for SPI-3 and SPI-5, which have complex evolutionary histories (Hensel 2004).

In contrast to *Salmonella*, where the successive acquisition of virulence genes and PAIs along a single evolutionary line leads to the development of a group of relatively similar pathogens, the evolution of *E. coli* is different and many distinct pathotypes are known within this species. Of particular interest are enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli*, which show a form of virulence evolution that is different from that seen with *S. enterica* and is called parallel evolution (Reid et al. 2000). The major virulence genes of EPEC and EHEC are located on a PAI called locus of enterocyte effacement (LEE), on plasmids (EAF-plasmid and EHEC-plasmid, respectively) and lambdoid phages (the Shiga toxin- or Stx-phages of EHEC). The LEE is responsible for the intimate adhesion of EPEC and EHEC to the intestinal epithelium and the reorganization of the cytoskeleton leading to attaching and effacing (AE) lesions. The EAF plasmid encodes many factors including a bundle forming pilus and regulatory factors for the expression of the LEE. The EHEC plasmid encodes different factors from those on the EAF plasmid, including diverse toxins probably involved in the development of hemorrhagic colitis and hemolytic uremic syndrome.

Phylogenetic analysis of *E. coli* populations shows that, despite their clonal nature, the distribution of virulence factors of EPEC and EHEC does not entirely fit a simple clonal evolution similar to *Salmonella* (Wieler et al. 1997; Reid et al. 2000; Donnenberg and Whittam 2001). For instance, The LEE and its variants are found in more than four major but phylogenetically unrelated *E. coli* lineages (Reid et al. 2000; Lacher et al. 2007). The whole PAI has been introduced separately and repeatedly in the chromosome of these different lineages. The variable chromosomal location of the LEE in different *E. coli* lineages and the mosaic structure of some of its components support this hypothesis (see below). Furthermore, subsequent to the acquisition of the LEE, the EAF plasmid was acquired separately in several lineages (Lacher et al. 2007), thus leading to the repeated parallel emergence of several unrelated EPEC lineages. Finally, a similar scenario seems to have happened with the EHEC plasmid and the Stx phages, which were also

acquired several times in different *E. coli* lineages. This led to the repeated independent emergence of EHEC in at least two unrelated *E. coli* lineages (EHEC 1 and EHEC 2).

The mobility of the LEE is further supported by its presence in bacterial species other than *E. coli*, including *Citrobacter rodentium* (McDaniel et al. 1995). Similarly, the presence of plasmids related to the EHEC plasmid in numerous phylogentically unrelated STEC lacking the LEE or even in nontoxigenic *E. coli* demonstrates the horizontal spread of this plasmid. In the case of the Stx phages, their widespread presence in the environment, demonstration of their mobilization *in vitro*, and diversity in clonally related EHEC show that they are still actively moving within *E. coli* populations. Thus, in contrast to the case of *Salmonella*, ordered acquisition of virulence genes through HGT has taken place in parallel repeatedly in *E. coli* populations. This shows the strong plasticity and adaptability of pathogens but also demonstrates that the constraints to which they are submitted by their host and their environment put some limits on the possible strategies available for the colonization of new ecological niches.

The LEE in EPEC and EHEC is also a good example of the different effects of selection on the microevolution of virulence factors and on the emergence of mosaic structures. Phylogenetic analyses suggest that after its formation through the amalgamation of several major clusters of genes, the whole island kept moving as a block between organisms. However, because of the different functions and exposure to the external environment and host selective pressure, its components evolved differently (Castillo et al. 2005). Because of functional constraints acting on their products, some genes, such as those for the TTSS encoded by the LEE, are submitted to purifying selection (selection against amino acid substitutions and against emergence of new variants), whereas others may be subjected to diversifying selection (selection in favor of substitutions and the emergence of new variants). This same picture is visible even within the different parts of some genes, such as the *eae* gene for the adherence factor intimin. This adherence protein consists of three domains: the N-terminal periplasmic domain, the central transmembrane domain, and the C-terminal extracellular domain. The N-terminal and central domains appear to be much more conserved than the C terminus. A comparison of synonymous and nonsynonymous mutations in the domains of the intimin gene shows that, in order to maintain its functionality, a purifying selection is at work on most of the molecule. However, despite this general purifying selection, diversifying selection seems to affect the C-terminal domain and to promote amino acid replacements associated with changes in charges in this exposed part of the molecule (Tarr and Whittam 2002). This variability is thought to allow the pathogen to circumvent the immune response of host populations and to explore new ecological niches by adapting to new host cell receptors or to other host species. Thus, the C-terminal domain of intimin provides an illustration of the diversifying selection generally acting on many secreted or surface-exposed virulence factors.

Phylogenetic analysis has shown that HGT within *E. coli* populations and/or between *E. coli* and other bacterial species followed by recombination events is responsible for the mosaic structure observed in the intimin gene and for the higher diversity of the extracellular domain (McGraw et al. 1999). Thus, not only has the LEE as a whole been transferred horizontally and inserted in the chromosome of different bacterial lineages, but also recombinations between LEEs of different origins have taken place in the evolution of EPEC and EHEC.

Listeria and *Bacillus*: Gains and Losses on Clonal Evolutionary Frames

Listeria are widespread saprophytic inhabitants of soil and decaying vegetation, but two of the six species of this genus (*L. monocyogenes* and *L. ivanovii*) are also opportunistic pathogens of animals and humans. Numerous virulence genes and two pathogenicity islets, called LIPI-1 and LIPI-2, have been described for these two species. The latter is found only in *L. ivanovii*, but LIPI-1 is found in both pathogenic species and the nonpathogenic species *Listeria seeligeri* (Vazquez-Boland et al. 2001). Interestingly, the LIPI-1 of *L. seeligeri* seems to contain the complete set of virulence genes required for pathogenicity, but their expression has been corrupted by insertions within the PAI.

Phylogenetic analysis of the genus shows that the three carriers of LIPI-1 are not more closely related to one another than to *Listeria welshimeri* and *Listeria innocua*. This distribution would, at first glance, suggest a repeated horizontal transfer of LIPI-1 within the *Listeria* genus. However, the insertion site of LIPI-1 is the same in all three

species, and its G + C content and codon usage are similar to those of the core genome of *Listeria* species, suggesting that the acquisition of LIPI-1 is a relatively old event in the evolution of *Listeria*. This PAI was probably inserted only once into the genome of *Listeria* after *Listeria grayi*, and the common ancestor of the other *Listeria* species diverged. The acquisition mechanism of LIPI-1 is not clear but may have involved phage transduction or movement through transposons (Cai and Wiedmann 2001).

LIPI-1 may have been of advantage as a defense against phagocytosis by soil inhabitants and certainly for the colonization of the new ecological niche represented by the cells of vertebrates. It was subsequently lost and/or inactivated in the nonpathogenic species for which it was not of significant selective advantage (Vazquez-Boland et al. 2001). A relatively similar phenomenon may have occurred with a gene cluster associated with the internalin genes *inlA* and *inlB* (Buchrieser 2007). The genus *Listeria* therefore represents a good illustration of the trial and error game played in the evolution of microorganisms, in this particular case, for the acquisition and loss of virulence characteristics. It also gives some insights on how intricate the evolution of virulence is and how difficult it may be to reconstitute and model it *a posteriori*.

Listeria monocytogenes causes a variety of diseases in humans and animals, but all the *L. monocytogenes* strains are apparently equipped with the same basic arsenal of virulence genes encoded by LIPI-1. However, the *L. monocytogenes* species is composed of three main lineages, and the frequency of recovery of these lineages and of some clones within these lineages from clinical cases or from food or the environment varies significantly. Some clones are more prone to cause disease or outbreaks, whereas others seem to be better adapted to particular environments and food but less frequently associated with disease. It is not clear which factors are responsible for these adaptations, but the acquisition of new biochemical pathways for adaptation to specific environments and differences in the extent and diversity of the arsenal of cell surface components, such as the internalin family, have been suggested to play a role in virulence level variation (Buchrieser 2007). Thus, similarly to what happened with host adaptation in *S. enterica* subspecies I, a whole range of clonal adaptations to specific ecological niches has developed within a single relatively homogeneous bacterial species. This represents a common theme in the evolution of pathogens (Musser 1996).

The *Bacillus cereus* complex is an interesting example of how HGT can confuse our understanding of phylogeny in the face of very different pathotypes. On the basis of phenotypes, this complex is divided into three major species with very different natural histories (Jensen et al. 2003). *B. cereus*, the archetype of the group, is a common environmental bacillus often associated with food poisoning and occasionally with invasive diseases in humans and animals. *Bacillus thuringiensis* is a widespread bacillus producing deadly toxins for insects and often used for biological pest control. Finally, *Bacillus anthracis* is a highly pathogenic organism affecting primarily herbivores and not known to multiply efficiently outside of its hosts under natural conditions.

Despite their very different behaviors and host ranges, these three bacilli are highly related and should be regarded as pathotypes of a single species (Helgason et al. 2000). Indeed, the cereus group is composed of three major clades (Priest et al. 2004), but these clades do not correspond exactly to the current species. These three clades can be further divided into at least eight lineages. *B. anthracis* seems to be restricted to a single major clade, which also contains a majority (but not all) *B. cereus* strains. The second clade consists mainly (but not only) of *B. thuringiensis* strains, and the third clade is associated almost exclusively with nonpathogenic bacilli of other species. With the exception of those causing anthrax and to a certain extent those causing vomiting through production of emetic toxin, the different pathotypes of the cereus group (i.e., those associated with invasive infections and those associated with diarrhea) are not restricted to a single lineage (Didelot et al. 2009). This illustrates the presence of limited but biologically important HGT acting in the cereus group, on top of a relatively clear clonal structure.

Type III Secretion Systems and Flagella: Divergent Evolution

TTSSs were first found in *Yersinia* species. They were subsequently described in a large variety of gram-negative pathogens from animals (for instance, in EPEC and EHEC, *S. enterica*, *Shigella flexneri*, *P. aeruginosa*, *Burkholderia pseudomallei*, *Bordetella pertusis*, *Aeromonas salmonicicda*, and

diverse *Chlamydiaceae*) and from plants (*Erwinia amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Xanthomonas campestris*). TTSSs were first thought to be strictly associated with virulence and certainly represent one of the major and most fascinating virulence mechanisms in bacteria (for a review, see Hueck 1998). However, they have since been found in several animal and plant symbionts, such as *Rhizobium* sp., *Sodalis glossinidus*, and *Photorabdus luminescens* (Dale et al. 2002), and are now thought of as a widespread communication system between prokaryotic and eukaryotic cells (Foultier et al. 2002).

TTSSs typically consist of more than 20 components that form a needle-like injection system, on top of an energy-dependent transmembrane secretion system. Upon tight contact between the bacteria and the eukaryotic host cells, effector molecules are translocated from the bacterial cytosol into the host cell cytosol through the TTSS. The general structure of TTSS is similar in all species, but the translocated effector molecules vary drastically from one species to the other, leading to very different effects on the host cells and, therefore, to a broad variety of diseases. As may be expected, some parts of the TTSS may be the object of purifying selection in order to conserve their functionality and others (those exposed on the surface of the bacterial cell) are under the pressure of diversifying selection (Stavrinides et al. 2008). New effectors secreted by the TTSS injectisome seem to emerge through recombination between the regulatory and secretion/translocation modules of existing TTSS effector genes and random new effector modules, thus leading to the appearance of very diverse mosaic effector genes.

The genetic organization of TTSS genes is variable, but some blocks of genes are relatively conserved between bacterial species (syntheny) and allow the delineation of at least seven phylogenetically based broad families of TTSS (Troisfontaines and Cornelis 2005). The distribution of these TTSS families does not fit with the general phylogeny of the bacteria. Some species, such as *S. enterica*, even contain two TTSSs of different families (Foultier et al. 2002). TTSSs are usually located on mobile elements, such as plasmids and phages, or within PAIs. In addition, the G + C content and codon usage of TTSS genes are generally different from those of the core genome of the bacteria. Thus, as for many other virulence factors, HGT has played a major role in the evolution and spread of TTSS in bacterial populations (Troisfontaines and Cornelis 2005).

TTSSs show strong structural and genetic homologies with flagella. It is still not clear whether TTSSs are derived from flagella or if they both derive from an older common ancestor. However, a variety of genetic evidence supports the latter hypothesis (McCann and Guttman 2008) and suggests that TTSS and flagella represent a wonderful example of divergent evolution. The ancestral origin of TTSSs is not clear, but they may have first emerged in plant pathogens or in a *Chlamydia* ancestor. The G + C content of TTSS resembles that of the core genome of *Chlamydia* and, in contrast to other bacterial groups, where TTSSs are found only in some species or even only in some clones within one species (a potential sign of recent acquisition), they are found in practically all the *Chlamydia* investigated. The TTSSs of the *Chlamydia* species also share more homogeneity than those of other groups of related species. In addition, the TTSS genes of *Chlamydia* are dispersed in the genome and not tightly clustered as in the Proteobacteria. All these observations suggest that TTSSs were not acquired recently by *Chlamydia* through one single HGT as in other bacterial species but have been present for a very long time in *Chlamydia* and may find their origin in this phylogenetic lineage (Kim 2001).

Evolution of Obligate Intracellular Pathogens: What Happens with Unnecessary Genetic Material

At first glance, only a minority of pathogens seem to have acquired the ability to survive within host cells. However, on closer examination it is evident that many pathogens (and commensals) have the ability to survive intracellularly at some point in their encounter with their hosts. Some researchers have even suggested that this is probably a natural consequence of the environmental origin of most bacterial pathogens that had originally to survive their encounter with protist predators (Casadevall 2008). Such a phenomenon is clearly observable in the case of the opportunistic pathogens of the genus *Legionella* and their natural amoeba hosts.

A number of strategies have been conserved or further developed by some bacterial pathogens to specifically survive in the hostile intracellular environment and exploit this niche in their hosts in order to spread and multiply. However, the vast majority

of intracellular pathogens have kept the ability not only to survive but also to multiply outside their host cells. Only a few bacteria, including, for instance, *Rickettsiae* and *Chlamydiae*, followed this path further and adopted the obligate intracellular lifestyle either as pathogens or as symbionts. At the very end of this spectrum, some have even completely lost their own identity to become vertically transmitted cellular organelles, such as mitochondria, which are thought to have derived from *Rickettsia* ancestors (Andersson and Kurland 1998).

It is one of the basic rules of Darwinian evolution that, to become fixed in a population, a characteristic should be associated with higher fitness and therefore be of advantage to single organisms and/or the population as a whole. Characteristics that are of no use to the community will tend to disappear in the course of evolution. This rule is also true for bacteria. Thus, depending on its usual environment, a pathogen may tend to lose some characteristics, which would be of advantage under other unlikely conditions. Generalist opportunistic pathogens, such as *P. aeruginosa*, confronted with many different environments, will tend to require more variable characteristics and will therefore tend to have a larger genome than organisms restricted to a specific environment or host. Extremely specialized organisms, such as primary symbionts and obligate intracellular pathogens, will need less flexibility and therefore less genetic material. They also often tend to take advantage of metabolic products and energy sources of their host. For instance, amino acid, purine, and pyrimidine biosynthesis pathways are frequently lost in obligate intracellular pathogens (some transport mechanisms may have to be acquired to compensate for these losses). In these organisms, motility-related structures, such as flagella, also tend to disappear or to evolve into secretion systems, as has been shown for *Chlamydia*. Loss of phenotypic functionality goes in parallel with gene inactivation through mutations and loss of genetic material, resulting in genome size reduction, a hallmark of obligate intracellular organisms (Andersson and Kurland 1998). Multiple examples of such genomic reductions also called genome decay have emerged with the availability of genome sequences of obligate pathogens and of obligate intracellular pathogens in particular. As a consequence of the accumulation of nonlethal mutations, a larger proportion of noncoding sequences is present in the genome of many of these organisms in comparison to extracellular bacteria. In addition, they also tend to show a scrambled genome structure resulting from the homologous recombination between repeated sequences which are thought to lead to the loss of genetic material and genome size reduction.

Genome decay represents a particularly dangerous evolutionary path for a pathogen population because it is frequently unidirectional and, in contrast to the situation for bacteria with an extracellular phase, there is no way back. The intrinsic intracellular isolation of a pathogen limits the availability of HGT, and lost or altered genetic material and biological functions cannot be restored easily, thus ending in extinction of numerous clonal lineages within a population at every selection bottleneck (for more details, see "Muller's ratchet" in Andersson and Kurland 1998).

Small genomes and losses of similar biological functions have been found in completely unrelated bacterial phyla, thus confirming that they are the result of convergent evolution and represent an evolved and not an ancestral state. Under the simplistic explanation of elimination of useless genes, one would expect that the genes remaining in small genomes would correspond to a few vital functions similar in all these unrelated bacteria. Essential functions, such as those necessary for DNA replication, transcription, and translation, are those that would remain conserved in these organisms. This is apparently not always the case, and much fewer than the total number of genes vital to the survival and multiplication of a microorganism are common to these diverse reduced genomes (Moran 2002). Once again, common wisdom does not work and evolutionary paths are often more sophisticated and tortuous than expected. Redundancy in ancestral genomes has allowed for several alternatives in each step of the genome reduction and each obligate pathogen has consequently followed its own way of genome decay, depending on the first critical steps in the reduction process. Bottlenecks (periods of small-sized populations) in the transmission chain of obligate pathogens and the consequent lack of a diversified population for selection are thought to have led to this kind of stochastic evolution.

To add to the complexity of genome decay, the loss of some functions may, under particular circumstances, be of advantage to the pathogen (Dobrindt and Hacker 2001) and some reduction steps may therefore not be completely stochastic.

The transition of *Yersinia pseudotuberculosis* to *Yersinia pestis* and the emergence of *Shigella* spp. and enteroinvasive *E. coli* from nonpathogenic *E. coli* are examples in which antivirulence genes were probably counterselected. In both instances, the loss of some metabolic functions was associated with an increase in virulence of the pathogen. In the case of *Shigella*, lysine decarboxylase produced by the *cadA* gene inhibited the function of a plasmid-encoded enterotoxin. The loss of *cadA* seems to represent such a great advantage for the pathogen that it happened repeatedly in at least four bacterial lineages (Maurelli 2007).

CONCLUSION

Recent technologies are shedding new light on the evolution of virulence through the window of comparative genomics. The more we learn about them, the more bacterial pathogens surprise us with their complex responses to the challenges they encounter in their life cycle. Their high reproduction and evolution rates provide them with the tools to rapidly adapt to new environments and hosts. The constant changes and intensification we are imposing on animal husbandry and on the environment also bring changes in the world of bacterial pathogens. More and more infectious diseases are becoming multifactorial and polymicrobic, which will certainly lead to unexpected synergies and coevolution mechanisms between the agents involved in such infections. New approaches, such as metagenomics, transcriptomics, and proteomics, will help us not only to describe these complex communities but also to understand better their dynamics and how they evolve.

The human responses to bacterial diseases are multiple. In the first place, we try to cut transmission chains and reduce exposure to bacterial pathogens. As discussed earlier in this chapter, this may have long-term consequences on the evolution of virulence that remain to be appreciated completely. In addition to this approach, immunization is used massively to prevent some infectious diseases. A number of examples demonstrate how pathogens can adapt to this challenge by changing their antigenic properties. Thus, although usually very successful on a short-term basis, this approach will probably remain in a number of cases a continuous innovation race between humans and pathogens.

Finally, infectious disease prevention and treatment with antimicrobial agents is the third major approach we take to reducing the burden of infections. However, the intensive and sometimes indiscriminate use of antimicrobial agents may have very strong implications in terms of bacterial evolution. Pathogens have developed numerous and fascinating coordinated answers to survive the challenges encountered in their host. Antimicrobial agents represent only one more challenge bacteria encounter in this context. They respond effectively to it by rapidly developing and acquiring numerous antimicrobial resistance mechanisms. Some pathogens already show physical linkages of virulence and resistance determinants on mobile genetic elements in the same way they gather (and coordinate) multiple virulence determinants on mobile elements. It will therefore be of great interest to observe how the use of antimicrobial agents will influence not only the evolution of antimicrobial resistance but maybe also virulence.

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4
Streptococcus

J. F. Timoney

INTRODUCTION

Streptococci are gram-positive spherical bacteria less than 2 μm that typically grow by cell division in one plane, so that nascent cells form a linear array. Most are facultatively anaerobic and catalase negative with complex and variable nutritional requirements, which reflect adaptation as commensals or parasites. Identification is based on colony characteristics; hemolytic properties; carbohydrate and protein antigen composition; fermentation and other biochemical reactions; DNA sequence of 16 and 23S rRNA genes; and, within species, multilocus enzyme electropherotype and multilocus sequence typing (MLST).

The majority of pathogenic streptococci possess a serologically active carbohydrate antigenically different from one species or group of species to another. These cell wall antigens, designated A-H and K-V, are the basis of the Lancefield grouping system and are widely used by clinical laboratories for serogrouping. The antigens are extracted by autoclaving, formamide treatment, or enzymic digestion for testing with commercially available sera. Groups B, C, D, E, G, L, U, and V contain the pyogenic streptococci responsible for suppurative (pus-producing) infections in a variety of host species. Some pathogenic streptococci, notably *Streptococcus uberis*, *Streptococcus parauberis*, and *Streptococcus pneumoniae*, are not groupable in the Lancefield scheme and are identified by features such as fermentation behavior, ability to grow at different temperatures, salt tolerance, optochin sensitivity, bile solubility, and 16S rRNA gene sequences.

The virulence factors of the streptococci most frequently involved in animal disease are shown in table 4.1. With the exception of *S. pneumoniae* and *S. suis*, all are loosely categorized as pyogenic. Comparison of the available genome sequences of the pyogenic streptococci shows that about 66% of their genetic content is common to all; the remainder is variable and formed by genes associated with prophages, integrative conjugative elements (ICEs), insertion elements (ISs), and other genes acquired by horizontal transfer (Beres et al. 2008). In general, streptococcal virulence is based on surface and secreted proteins and on structures that directly or indirectly impede phagocytosis, are involved in adhesion and carbohydrate metabolism, or induce release of pro-inflammatory cytokines. The best understood streptococcal virulence factors are the hyaluronic acid capsule, the antiphagocytic M proteins, and the pyrogenic exotoxins. However, other molecules, including streptolysins, proteases, leukocidal toxins, plasminogen activators (streptokinase), and possibly plasmin receptors found on the surface or secreted, also contribute to pathogenicity. In addition, most pathogenic streptococci have the ability to bind components of the host's plasma, such as albumin, immunoglobulins, and fibrinogen, and to bind to fibronectin, laminin, and other components of the host cell. Organisms coated with one or more of these plasma components may be able to evade host defenses either by escaping detection or by blocking deposition of opsonic components of complements.

The streptococci pathogenic for domestic animals can be grouped by their adaptation to specific organs/body systems. Thus, *S. agalactiae*, *S.*

Table 4.1. Pathogenic Streptococci of Animals

Species	Lancefield group	Virulence(-associated) factors	Disease
S. agalactiae	B	Capsular polysaccharide; C, R, and X proteins; CAMP factor; hyaluronidase; lipoteichoic acid; proteases; CspA; collagenase; D-alanylated lipoteichoic acid; neuraminidase	Mastitis
S. dysgalactiae subsp. *dysgalactiae*	C	Hyaluronidase; streptokinase; fibronectin binding proteins fnb A and B; protein G; plasminogen receptor; streptodornase; M-like proteins; alpha-2-macroglobulin receptor	Mastitis
S. dysgalactiae subsp. *equisimilis*	A, C, G, L	As for subsp. *dysgalactiae* but also including streptolysin S and O	Porcine arthritis; pneumonia in kittens and puppies. lymphadenitis; metritis; placentitis in *Equidae*
S. equi	C	Capsular hyaluronic acid; antiphagocytic SeM, Se18.9 and IdeE; streptolysin S; pyrogenic exotoxins: streptokinase; peptidoglycan; fibronectin binding protein; proteases; tonsil binding SzPSe and Se51.9; streptokinase; equibactin	Equine strangles
S. zooepidemicus	C	Capsular hyaluronic acid; streptokinase; proteases; streptolysin S; peptidoglycan; tonsil binding SzP protein; fibronectin binding proteins; IgG binding protein	Opportunist pyogen; pneumonia; metritis; joint ill
S. suis		Capsule; MRP and EF proteins; suilysin; OFS, enolase, SAO, adhesins.	Meningoencephalitis; septicemia and arthritis in pigs
S. porcinus	E, P, U, V	M protein; streptokinase	Porcine cervical lymphadenitis
S. canis	G	M protein; streptolysin O	Canine and feline metritis and vaginitis; neonatal bacteremia of kittens; lymphadenitis of juvenile cats, guinea pigs, and rats
S. uberis		Secreted antiphagocytic factor(s); casein receptor; hyaluronidase; CAMP-like *uberis* factor; mammary cell adhesin; plasminogen activator PauA; Mr scavenger MTuA	
S. pneumoniae	–	Capsular polysaccharide; neuraminidases; pneumolysin; autolysin; IgA protease; fibronectin binding proteins; peptide permeases; ZmpB metalloproteinase; choline binding proteins PsPA, LytA and CppA	Broncheolitis and pneumonia in horses in training

dysgalactiae, and *S. uberis* cause disease in the udder; *S. equi*, *S. canis* (some M types), and *S. porcinus* are pathogens of the lymphatics of the head and neck; *S. pneumoniae* causes lower respiratory tract disease in horses; *S. suis* is adapted to survive on/in blood mononuclear cells that transport it to the central nervous system (CNS), lungs, and joints. Moreover, all these streptococci exhibit varying degrees of host specificity and so contrast with the unlisted *Streptococcus zooepidemicus*, which, although closely related to *Streptococcus equi*, is an opportunity pathogen of different body systems in a variety of hosts.

STREPTOCOCCUS AGALACTIAE

Streptococcus agalactiae, the lone member of the Lancefield group B, is an important cause of chronic, contagious bovine mastitis. It is also a cause of mastitis and invasive disease in camels and an occasional cause of disease in dogs, cats, fish, and hamsters. A serious pathogen of human newborns, it causes neonatal septicemia and meningitis. Human and bovine populations of *S. agalactiae* are distinct and separate, and there is only limited evidence of interspecies transmission (Sukhanand et al. 2005). However, human isolates of hypervirulent clonal complex 17 are derived from an ancestor of bovine origin. Salicin and lactose fermentation and bacteriocin and bacteriophage typing are helpful in differentiating populations from humans and cattle.

There are nine serotypes based on a polysaccharide capsule which varies based on the arrangement of four sugars into a unique repeated unit. Horizontal transfer of genes for capsule biosynthesis accounts for diversity in capsule serotype. Capsule type Ia constitutes about 70% of bovine isolates in the New York State, but different types may be numerous in other geographic areas (Norcross and Oliver 1976). About 25% of strains are untypable.

Conjugative transfer of large segments of chromosomal DNA among strains of *S. agalactiae* (Brochet et al. 2008) accounts for both strain diversity and emergence of clonal complexes with combinations of virulence factors and capsular type suited to particular host niches.

Streptococcus agalactiae is an obligate parasite of the epithelium and tissues of ruminant mammary glands, and eradication of the organism from herds is therefore possible by identification of animals with mammary infection followed by treatment or culling. In contrast, *S. agalactiae* is primarily a commensal of the human gastrointestinal and genitourinary tracts.

Virulence Factors

Much of the information on potential virulence factors of *S. agalactiae* has been derived from studies on human isolates in mouse and rat models and therefore must be cautiously interpreted in the context of bovine mastitis. Bovine isolates generally have different properties from those of their counterparts from humans and usually lack well-studied virulence factors, such as ScpB, Lmb, and IgA binding β protein. Capsular polysaccharide, including its type-specific antigen, is antiphagocytic, and specific antibodies are mouse protective and contribute to resistance of human infants to infection.

Terminal sialic acid on type III capsular polysaccharide inhibits activation of the alternate complement pathway and blocks deposition of C3 on the bacterial surface. The capsule also increases the affinity of the complement control factor H for C3b bound to the surface of the cell wall and thereby reduces both the activity of the C3 convertase and further deposition of C3b on the cell (Marques et al. 1992).

Although less capsular polysaccharide is expressed on bovine than on human strains, the former are nevertheless capable of activating the alternate pathway. Moreover, bovine antibodies to the group B polysaccharide fix the complement and C3 via the classical pathway (Rainard and Boulard 1992).

A diverse and large number of proteins, often encoded on pathogenicity islands, are coordinately expressed with the capsule on the surface of *S. agalactiae*. They function in adhesion, invasion, iron binding, metabolism, transport, and inhibition of phagocytosis (Lindahl et al. 2005). The best studied group is the Alp family of proteins, which constitute part of the C antigen. These proteins are trypsin resistant, cluster in molecular mass between 100 and 120 kDa, and contain series of long tandem repeats. A common structural feature is an Ig-like fold, suggesting a molecular recognition function. The four members of the family are designated α, Rib, R28, and Alp2. Their occurrence varies with the capsule type—for example, type Ia strains commonly express α protein. Their function is unknown, but the specific antibody is mouse protective.

The C antigen, in addition to one of the Alp proteins, also contains the trypsin-sensitive β protein. This protein interacts with human IgA-Fc and factor H. It lacks tandem repeats, but its C terminus is rich in prolines with a unique periodic sequence termed "XPZ." It elicits a protective antibody in mice.

Proteinaceous trypsin-resistant filamentous protusions from the cell surface have been described on *S. agalactiae* by Wagner et al. (1982) and may constitute the first sightings of the pilus-like structures described later by Malone et al. in 2005. The latter consist of three proteins, GBS 59, 80, and 104, two of which are protective in mice.

Another surface protein antigen of about 100 kDa, termed X, which occurs on many untypable bovine strains of *S. agalactiae* is of unknown significance in pathogenesis. This antigen is opsonic and apparently different from the cell surface Sas 97/104 protein (Wanger and Dunny 1987), which is immunodominant for the bovine and found on about 50% of bovine strains. Culture supernatant contains a similar sized protein (BPS) together with a 5′-nucleotidase, which reacts with IgG in whey of infected cows (Trigo et al. 2008). The presence or absence of Sas 97/104 did not affect bacterial virulence in a guinea pig model.

A serine protease, CspA, with homology to caseinases of lactic acid bacteria, cleaves fibrinogen, releasing the adhesive alpha chain (Harris et al. 2003). The alpha chain binds to the bacterial surface and impedes opsonophagocytosis.

The 105-kDa BPS protein (group B streptococcal protective surface antigen) is found predominantly with R1 on type 1a isolates and is protectively immunogenic for mice (Erdogan et al. 2002). The 45-kDa Sip protein is exposed on the polar surfaces of all serovars of *S. agalactiae*. Sip lacks an anchor sequence motif, and so its attachment to the bacterial surface may depend on an interaction with another bacterial protein. The immunogenicity of BPS or Sip for cattle has not been reported. Since Sip is conserved among all serovars, it is an attractive candidate for vaccine evaluation.

The CAMP factor is a 23.5-kDa ceramide binding protein of *S. agalactiae* that potentiates the action of staphylococcal sphingomyelinase (beta toxin). The lethal properties of the CAMP factor for cell cultures and for rabbits and mice suggest that it may have a cytotoxic action for mammary tissue. The protein binds to the Fc region of IgM and IgG. Insertional inactivation of *efb*, the gene that encodes this protein, increases mouse LD_{50} 50 times. Virulence of these mutants for the mammary gland has not been reported. Other potential virulence factors of *S. agalactiae* for the mammary gland include neuraminidase, hemolysin, vasoactive extracellular toxin, and D-alanylated lipoteichoic acid.

Streptoccocus agalactiae enters through the teat meatus, and colonization of the gland is favored by adhesion to the epithelium of the gland sinuses (Frost et al. 1977). Back-jetting of contaminated milk against the teat ends at milking time is an important factor in the introduction of infection past the teat sphincter. Keratin and associated bacteriostatic long-chain fatty acids of the teat canal are the first barriers to physical penetration of the epithelial lining. Bacterial multiplication is controlled by the lactoperoxidase–thiocyanante–H_2O_2 system, by lysozyme, and by the flushing action of milk during milking. Multiplication on the epithelium of the teat and duct sinuses results in a slowly progressing inflammation and fibrosis. Although *S. agalactiae* rarely penetrates the epithelium, some cows may experience a transient invasion during the first few days in which the organism enters the lymphatics and travels to the supramammary lymph nodes. Release of chemoattractants from damaged host cells and *S. agalactiae* attracts polymorphonuclear leukocytes (PMNs), which then ingest and kill many of the invading streptococci. Since normal milk has very low complement content and thus cannot itself serve as a source of C3, opsonization is probably derived from C3 in the inflammatory exudate, which becomes fixed on the bacterial surface following activation of the alternative complement pathway. Initial invasion is more likely to result in colonization in older cows and in mammary glands, where there is delay in the arrival of PMNs at the site of invasion. Death of PMNs and release of lysosomal enzymes cause further tissue damage and inflammation. Fibrin plug formation in the smaller milk ducts may lead to involution of secretory tissue and loss of milk-producing capacity ("agalactiae"). Without treatment, the organism persists in the face of the host's immune response, and the infection and mastitis become chronic. The antiphagocytic effect of capsular sialylated polysaccharide may be the important bacterial virulence factor in persistence.

Immunity

Much of the protective activity of colostrum against *S. agalactiae* has been shown to be associated with IgA and IgM. Serum antibodies appear to have little or no protective effect. Agglutinins in milk of infected cows, together with failure of bacterial clearance mechanisms in the udder, suggest that acquired immune responses are insufficient in

clearance. However, immunoglobulins specific for capsular polysaccharide may play a role in ameliorating the disease process, a conclusion reached by Norcross et al. (1968), who noted that clinical signs were often absent in experimentally infected cows with preexisting circulating antibody. They postulated that this antibody neutralized extracellular products of *S. agalactiae* involved in the inflammatory response. The immunoreactive BPS and 5′-nucleotidase are other promising vaccine candidates.

The antigenicity of group B polysaccharide is greatly increased by conjugation to proteins, such as ovalbumin. Immunization of cows with conjugate produced strong capsule polysaccharide-specific serum IgG1 and IgG2 responses (Rainard 1992). Other approaches to immunization of cattle include the use of surface glyceraldehydes-3-phosphate dehydrogenase protein (plasmin receptor, GAPDH) and a chimeric CAMP antigen composed of epitopes of the *S. agalactiae* and *S. uberis* CAMP factors (Fontaine et al. 2002). This combination has shown some promise as a subunit vaccine against *S. uberis* and *S. agalactiae* mastitis.

STREPTOCOCCUS DYSGALACTIAE

Streptococci with the species designation *dysgalactiae* belong to Lancefield groups A, C, G, and L and are found as epithelial and mucosal commensals of most mammals and birds and as opportunist pathogens of domestic animals. A reclassification, based on genotypic and phenotypic characterization, established the subspecies *dysgalactiae* and *equisimilis*. Strains in subsp. *dysgalactiae* are group C only, alpha hemolytic, and found mainly as a cause of acute and subclinical mastitis in cattle. Strains in subsp. *equisimilis* are betahemolytic and may have either the A, C, G, or L group antigens and cause disease in a variety of animal species including humans.

Virulence Factors

Both *S. dysgalactiae* subspecies express numerous surface-exposed and secreted proteins that bind to plasma or host tissue components. These streptococcal proteins include protein G, fnbA, fnbB, IgG, IgA, and α2 macroglobulin receptor Mig, plasminogen receptor (GAPC), and M-like proteins, which bind IgG, fibronectin, α2 macroglobulin, plasminogen, and fibrinogen, respectively (Vasi et al. 2000). Secreted proteins include streptokinase, pyrogenic exotoxin G (SPEG), streptolysin O or S, streptodornase (DNase), pyrogenic exotoxin G, and hyaluronidase.

The roles of these proteins in the pathogenesis of bovine mastitis are poorly understood. Surface coating with plasma proteins including immunoglobulins in combination with M-like proteins may serve to reduce phagocytosis. Immunization of cows with Mig and GAPC reduces cell counts in milk following challenge (Bolton et al. 2004). GAPC-immunized cows also showed reductions in the number of challenge bacteria in their milk. Release of hyaluronidase and fibrinolysin may be of value in tissue penetration and dissemination. Bovine sera contain an antibody specific for SPE-G, and the recombinant protein stimulates proliferation of bovine PBMCs. However, its significance in the pathogenesis of mastitis is unknown. Infections of the mammary gland are usually associated with damage, such as insect bites or other injury to the teat or udder epithelium, which would facilitate direct access of surface exposed or secreted virulence bacterial proteins to their targets in the host. Since infections are opportunistic, cases occur sporadically with an acute clinical course. *Arcanobacterium pyogenes* is frequently present as a synergist.

Streptococcus dysgalactiae subsp. *equisimilis* includes group G and L strains from humans, and C and L strains from animals. Isolations of subsp. *equisimilis* are made only infrequently from horses, cattle, dogs, and cats. It is most frequently isolated from joints of piglets that have acquired infection from sows that are tonsil carriers. Piglets are invaded via wounds, umbilicus, or tonsil and develop suppurative arthritis. Although there appear to be at least four M types of subsp. *equisimilis* from swine (Wood and Ross 1975), the role of M protein-specific antibody in virulence and protection is unknown. Subsp. *equisimilis* has occasionally been isolated from abscessed lymph nodes of horses (Laus et al. 2007) and from aborted placentas. The streptokinases of subsp. *equisimilis* show strict species-specific plasminogen activation (i.e., porcine isolates activate porcine but not equine plasminogen) (McCoy et al. 1991).

STREPTOCOCCUS UBERIS

Streptococcus uberis is a tonsillar, intestinal, mucosal, and epithelial commensal of cattle and responsible for about 20–30% of cases of clinical

mastitis in dairy herds in North America, Europe, and Australia. It is distinguished from the phenotypically similar but rarely isolated *Streptoccocus parauberis* by differences in its 16S and 23S rRNA genes and by the absence of genes for *pauA* and the oligopeptide permease OppF. Isolates of *S uberis* vary in MLST and in protein phenotype between and within infected herds, although persisting infections in individual cows are usually caused by a single genotype. Many infections are opportunistic invasions of the mammary gland of older cows under conditions of heavy environmental soiling with feces. Analysis of the genomic sequence of *S. uberis* reveals a great variety of metabolic capabilities and nutritional flexibility but relatively few classical streptococcal virulence factors (Ward et al. 2009). Thus, it is well equipped to cope with a variety of environments, such as those encountered as a commensal of the bovine intestine, an environmental contaminant, or a pathogen of the mammary gland.

Following entry through the teat canal, the organism attaches, proliferates, and induces an influx of neutrophils into the secretory acini that is evident in 24 h. This is followed by septal edema, vacuolation of secretory cells, necrosis of alveoli, and infiltration of septa by lymphocytes. As the disease progresses, there is hypertrophy of ductular epithelium, involution of glandular tissue, and early stage fibrosis. Streptococci are free and within alveolar epithelial cells and macrophages in the alveolar lumina but are infrequent in neutrophils (Thomas et al. 1994). The organism is also present in lymphatic vessels and lymph nodes and attaches to ductular epithelium. The severity of the mastitis varies greatly and is determined by strain virulence, number of infecting organisms, season, immune status of the cow, parity, and stage of lactation. Infections in some herds are more common following drying-off and more prevalent in cows with open teat canals. Cows with chronic infections often show a very slight inflammatory response and a normal humoral immune response.

Virulence Factors

Resistance to phagocytosis and the bactericidal activity of neutrophils is a hallmark of *S. uberis* infection. A hyaluronic acid capsule protective against phagocytosis and intracellular killing is expressed on a small percentage of isolates—the majority of isolates of *S. uberis* do not produce mucoid colonies, and so the capsule is not an essential virulence factor. Furthermore, *hasA* or *C* gene deletion mutants, although less resistant to phagocytosis by bovine neutrophils (Ward et al. 2001), are nevertheless pathogenic following entry into the mammary gland. Resistance to the bactericidal activity of neutrophils may be due to factors released by *S. uberis* as it replicates in milk. In addition, binding of casein to the bacterial surface increases resistance to phagocytosis (Leigh and Field 1994). Other potential virulence factors include hyaluronidase, a 28-kDa uberis factor similar to the CAMP factor of *S. agalactiae*, an adhesin specific for cubic mammary gland cells (Lammers et al. 2001), the plasminogen activator PauA (Rosey et al. 1999), and the manganese scavenger lipoprotein MtuA. Activation of plasmin may be important in the generation of essential amino acids from casein and in uncovering target sites for adhesins expressed on the bacterial surface.

Streptococcus uberis enters and survives within mammary epithelial cells for extended periods (Oliver et al. 1998). A novel adhesion molecule, SUAM (112 kDa), with lactoferrin-binding abilities, contributes to attachment to cells by serving as a bridge between epithelial receptors and the bacterium. Antibodies to this protein inhibit bacterial attachment and internalization. Similar molecules have been reported on other streptococcal mastitis pathogens.

Immunity

Specific immune responses have been shown to be involved in clearance of *S. uberis* from experimentally infected udders that had recovered from a previous infection (Hill 1988). Sera from infected cows react strongly with *S. uberis* proteins of 40–41, 59–65, and 118–122 kDa (Groschup et al. 1991). Antibodies of unknown clearance function begin to appear 3 days after challenge. Earlier studies indicate that milk becomes opsonic for *S. uberis* after mammary infection. A trypsin-resistant protein of 65 kDa has been shown to stimulate opsonic antibodies (Groschup and Timoney 1992). Also, milk of previously infected mammary glands inhibits growth of *S. uberis* (Fang et al. 1998). Nevertheless, protection against infection is not dependent solely on opsonic activity and neutrophils. Vaccination with culture filtrates rich in PauA, the plasminogen activator or streptokinase from *S. uberis*, induces protection in about 50% of challenged quarters of

immunized cows. Protection is correlated with levels of PauA-specific inhibitory antibodies (Leigh et al. 1999). Interestingly, *pauA* appears to have been acquired by horizontal transfer and is undergoing positive selection pressure that is possibly immunological (Zadoks et al. 2005).

The Gap C protein and the uberis CAMP factor have also shown some protective efficacy in vaccination-challenge trials in dairy cows (Fontaine et al. 2002).

In summary, the available data strongly suggest that effective immunity against *S. uberis* is multifunctional and involves a combination of immunogens.

STREPTOCOCCUS EQUI

Streptococcus equi causes strangles, a highly contagious infection of the upper respiratory tract and associated lymph nodes of solipeds. Isolates show remarkable antigenic conservation (Galán and Timoney 1988) and constitute a clone or biovar of the closely related *S. zooepidemicus*. This conclusion is based on comparison of genomic sequences, multienzyme electrophoresis, and 16S sRNA interspacer sequence studies (Holden et al. 2009). Nevertheless, polymorphism in DNA restriction fragments reveals unrepaired errors in DNA replication (Takai et al. 2000). Since small differences in nucleotide sequence may accumulate to the point of loss of or change in proteins encoded, continued survival of the clonal and highly host-adapted *S. equi* is dependent on a specific phenotype. Comparisons of the genome sequences of *S. equi* 4047 and of *S. zooepidemicus* H70 and MGCS10565 show that they are similar in size (2.25, 2.15, and 2.02 Mb, respectively) and share at least 96% DNA homology. Differences include the presence of four prophage sequences in *S. equi* that encode genes for pyrogenic exotoxins SePE-I, H, L, and M, hyaluronidase, phospholipase A2, muramidase, and equibactin E (a putative iron binding enhancing molecule) (fig. 4.1). In addition, some of the immunoreactive anchored and secreted proteins, such as SeM and Se18.9, encoded by genes with alleles in both *S. equi* and *S. zooepidemicus* differ in sequence with the consequence of changed or abolished function.

Two potentially functional pilus loci are found in the *S. zooepidemicus* genome but only one in *S. equi*. Other differences include many more copies of IS sequences in *S. equi* than in *S. zooepidemicus*, an expansion that has triggered DNA deletions/recombinations in sequences involved in lactose, ribose, and sorbitol metabolism. Genomic sequence comparisons have not so far revealed an explanation for the strict host adaptation of *S. equi* to equids. Given its strict host adaptation, the only source of *S. equi* is a nasal discharge or pus from an abscess, or feed, water, or fomite directly contaminated by a shedding equid. Clinically inapparent carriers, although infrequent, may be associated with persisting infection in resident horse populations or serve as vehicles of transfer to previously uninfected premises. Prolonged carriage is commonly associated with empyema of the guttural pouch or cranial sinuses from which the organism is intermittently discharged. Persisting infection of tonsillar tissue appears to be rare.

The incubation period varies from 3 to 14 days after exposure, and disease onset is marked by fever, lassitude, nasal discharge, slight cough, difficulty in swallowing, and swelling of the mandibular lymph nodes. Pressure of the enlarging retropharyngeal nodes and associated edema on the airway may cause respiratory difficulty, hence the common name of the disease. Metastasis may result in abscess formation in other locations, such as the lungs, abdomen, or brain. In older animals with residual immunity, strangles may present as the clinically atypical or catarrhal form. Strains of *S. equi* expressing bacteriophage-encoded hyaluronidase have also been associated with a clinically mild form of strangles.

Most cases recover quickly and uneventfully. Sequelae include myocarditis, anemia, purpura hemorrhagica, and glomerulonephritis. These latter two sequelae involve the formation of circulating immune complexes.

Virulence Factors

The known or putative virulence factors of *S. equi* include a nonantigenic hyaluronic acid capsule, hyaluronidase, streptolysin S, streptokinase, streptodornase, IgG Fc-receptor proteins, ADP-ribotransferase, pyrogenic exotoxins SePE-I, H, L, and M, peptidoglycan, antiphagocytic SeM, Se18.9 and IdeE, equibactin, and fibronectin-binding FNE and FNEB. A leukocidal toxin may also be produced.

Isolates of *S. equi* are almost always highly encapsulated and produce very mucoid colonies. Nonencapsulated mutants are much less virulent for mice and horses (Timoney and Galán 1985; Anzai

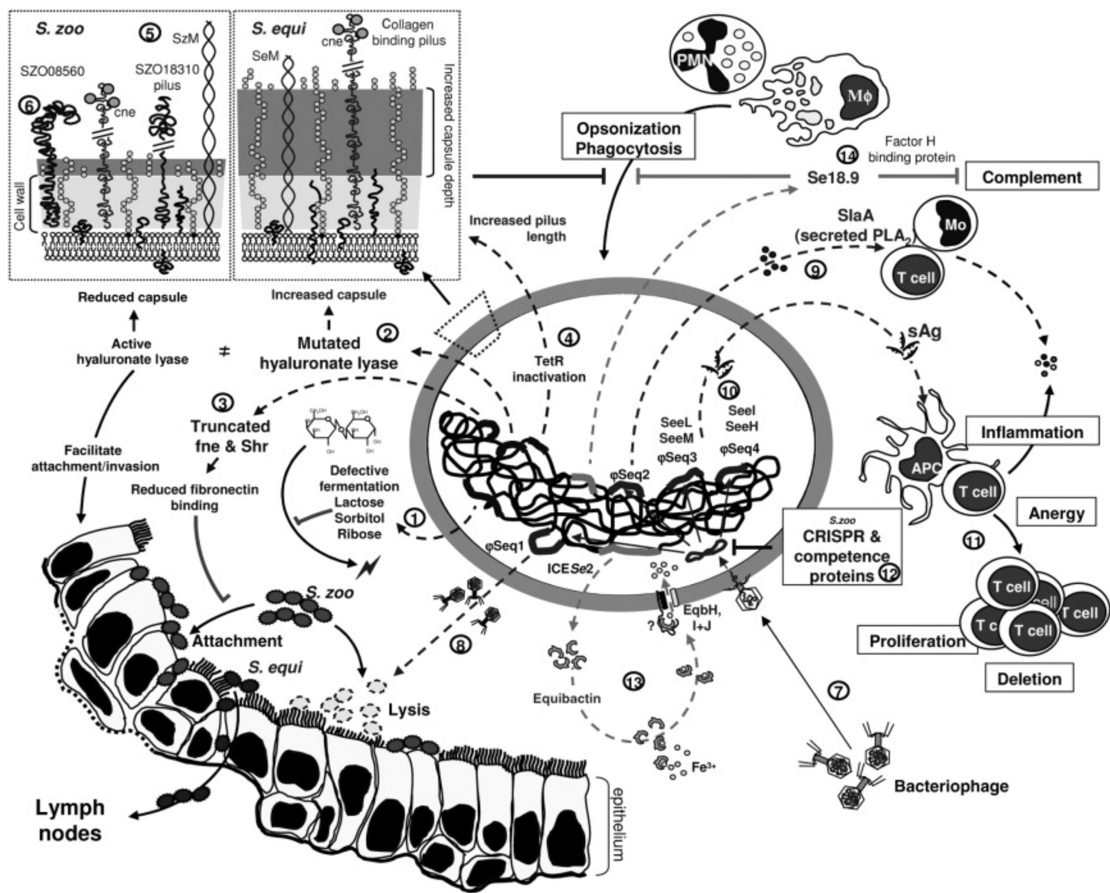

Figure 4.1. Hypothetical illustration showing genes and functions gained (red) and lost (blue) in the course of evolution of *S. equi* from the putative ancestral *S. zooepidemicus*. The figure is based on a comparison of the genome sequences of *S. equi* 4047 and *S. zooepidemicus* H70, and the legend is modified from that in figure 2 of Holden et al. (2009). (1) Loss of the ability of *S. equi* to ferment lactose, sorbitol, and ribose with possible adverse effect on its mucosal colonization ability. (2) Reduced hyaluronate lyase activity and abundant capsule synthesis by *S. equi* increases resistance to phagocytosis. (3) Truncation of FNE and SBR proteins in *S. equi* may decrease fibronectin-dependent attachment. (4) A nonsense mutation in the *tetR*-like regulator of *S. equi* may be associated with enhanced pilus production and adhesion to cell receptors in equids. (5) Loss of the SZO18310 pilus locus in *S. zooepidemicus* from *S. equi* may reduce its colonization potential in multiple hosts. (6) Loss of expression by *S. equi* of homologue of SZO08560 protein, which carries four Pfam repeat domains involved in host cell invasion by bacteria, such as *Listeria monocytogenes.* (7)–(12) Four prophages acquired by *S. equi* 4047 introduced genes for pyrogenic exotoxins SeeH, I, L, and M and phospholipase A2 (SlaA) that enhance its virulence and modulate inflammatory and immune responses in horses. Resistance of most *S. zooepidemicus* to phage integration is due to the presence of short palindromic nucleotide repeats (CRISPR) and associated genes. These sequences have been deleted from *S. equi* as a sequel to recombination between insertion (IS) elements. (13) Acquisition of the potential siderophore equibactin, encoded by the integrative conjugative element ICESe2, may be responsible for the high rate of proliferation of *S*. equi in tonsil and lymph nodes under conditions of low iron availability. (14) Binding of complement control factor H by Se18.9 reduces phagocytosis of *S. equi*. (See color plate)

et al. 1999). The hyaluronic acid capsule greatly reduces the number of streptococci that become associated with the surface of neutrophils and are subsequently ingested and killed. The capsule increases the negative charge and hydrophilicity of the bacterial surface and produces a localized reducing environment that protects oxygen–labile proteases. The capsule is also required for the functionality of SeM and possibly other surface-exposed hydrophobic proteins.

Streptokinase (Skc, plasminogen activator) released *in vivo* by *S. equi* interacts with the C-terminal serine protease domain of equine plasminogen to form active plasmin, a serine protease that hydrolyses fibrin (McCoy et al. 1991). A role for plasmin in the pathogenesis of strangles has not been proven, but its lytic action on fibrin and host matrix protein may aid in the spread and dispersion of the bacteria in tissue. Convalescent sera contain significant amounts of Skc-specific antibody. Other possible roles include *in situ* activation of complement and production of low-molecular-weight nitrogenous substrates for bacterial growth. The surface of *S. equi* has a receptor for plasmin.

The oxygen stable 36 amino acid oligopeptide, streptolysin S, is responsible for the betahemolysis produced by *S. equi*. Production of this bacteriocin-like cytotoxin is encoded by a nine-gene locus, and biologic activity requires stabilization by association with a carrier molecule. Binding to erythrocytes results in the formation of a transmembrane pore and irreversible osmotic lysis of the cell, a process similar to complement-mediated hemolysis. Damage to keratinocytes has also been noted. A streptolysin S mutant of *S. equi* had reduced invasive ability for horses (Timoney and Kumar 2008). Unlike *S. dysgalactiae*, subsp. *equisimilis* or *S. canis*, the closely related *S. equi* and *S. zooepidemicus* do not have a gene for streptolysin O.

A proteinaceous cytotoxic activity unrelated to streptolysin S has been detected in culture supernatant of *S. equi*. Equine PMNs incubated in the presence of culture supernatant showed signs of toxicity and became chemotactically unresponsive (Mukhtar and Timoney 1988). The toxin may target mitochondrial membranes because suspensions of equine PMNs exhibited intense respiratory activity shortly after exposure to culture supernatant, suggesting sudden release of respiratory enzymes. Nonhemolytic mutants of *S. equi* exhibited the same toxic effect, suggesting a toxic effect distinct from that of streptolysin S. A possible source of the toxic effect is the phage-associated phospholipase A2.

Peptidoglycan of *S. equi* is a potent activator of the alternative complement pathway, and chemotactic factors (C3a, C5a) released following incubation of peptidoglycan with plasma are strongly chemotactic for equine PMNs (Mukhtar and Timoney 1988). This is the basic pathologic process in strangles—the outpouring of PMNs in infected lymph nodes and tonsils and onto the upper respiratory mucosa. Peptidoglycan is also a potent pyrogen by inducing release of pyrogenic cytokines, such as interleukin-6 and tumor necrosis factor from leucocytes.

Genes for the pyrogenic exotoxins SePE-I, H, L, and M, acquired by phage-mediated transfer, may have been important in the formation of the clonal more virulent *S. equi* from its *S. zooepidemicus* ancestor (Artiushin et al. 2002). The pyrogenic mitogens have high immunomodulating capacity by binding simultaneously to the invariant region of class II MHC molecules on antigen presenting cells and to the variable region of the β-chain of the T cell receptor. The result is nonspecific T cell stimulation, proliferation, pro-inflammatory cytokine release, and production of an acute phase response with high fever, neutrophilia, and fibrinogenemia. These effects are characteristic of strangles and may be neutralized by antibody generated during convalescence or by active immunization with each mitogen.

The surface of *S. equi* potentially exposes 34 proteins anchored by their carboxy termini (LPXTG motif), and approximately 37 by their N termini (LYXC motif). In addition, proteins lacking these anchors may fix on the surface by other physicochemical interactions. The functions of most of the surface-exposed proteins are unknown. Based on sequence homology, some are adhesins, others have enzymic or transporter functions, and some, such as SeM, are antiphagocytic. Genes for immunogenic surface proteins show a tendency to be clustered in loci, suggesting coregulation or enbloc acquisition. For instance, the gene for SzPSe, a surface protein, is clustered with *CNE*, *Se51.9*, *Se46.8*, *Se44.2*, and *Se30.0*, genes for surface anchored proteins, and a sortase (Timoney et al. 2007). This gene cluster includes a locus that shares over 90% amino acid sequence identity with the Fim 1 locus of *S. zooepidemicus* MGCS10565. It is therefore likely that some of these proteins (CNE, Se51.9) are involved

in the pilus formation by *S. equi*. Interestingly, since the gene for SzPSe is immediately upstream of the operon encoding CNE and Se51.9, the tonsil-binding SzPSe may contribute to the attachment function of the pilus. It is even possible that a proteolytically cleaved fragment of SzPSe becomes part of the pilus structure since this protein is known to be present in a fragmented form in extracts of *S. equi* and *S. zooepidemicus*.

Binding of host plasma proteins to the bacterial surface is potentially an effective mode of concealment from host cellular recognition mechanisms. Bound proteins might also block access of C3 or specific antibody to target sites on the organism.

M proteins are antiphagocytic, acid-resistant, fibrillar molecules that project from the cell wall surface in an arrangement wherein two identical molecules are coiled around each other. A typical M protein molecule measures about 50–60 nm, with a long, coiled central region flanked by a short, random, coiled sequence at the N terminus and by a specialized, highly conserved arrangement of hydrophobic and charged amino acids at the C-terminal, anchor region. SeM of *S. equi* has a molecular mass of about 58 kDa.

Its antiphagocytic action is due to binding of fibrinogen to the N-terminal half and IgG to the central region (Boschwitz and Timoney 1994; Meehan et al. 2002). This interaction masks C3b binding sites on the bacterial surface and inhibits the alternative C3 and classical C5 convertases (Boschwitz and Timoney 1994a). Antibodies against specific linear epitopes override these effects and opsonize the streptococcus, so that it is effectively phagocytosed. Unlike the M proteins of *Streptococcus pyogenes*, SeM is highly conserved in size and sequence. However, variation in amino acid sequence in the N-terminal region of SeM has recently been detected in isolates from Japan, Europe, and North America (Anzai et al. 2005). These sequence variations do not affect opsonogenic epitopes and generally are absent from regions reactive with convalescent serum antibody. Some isolates from long-term guttural pouch carriers have in-frame deletions representing about 20% of the SeM gene between the signal sequence and the central repeat region (Chanter et al. 2000). Thus, the 5′ end of SeM may be unstable. Isolates with truncated SeM proteins were more susceptible to phagocytosis, but their virulence in horses has not been reported. Loss of SeM expression by *S. equi* results in loss of virulence but not of infectivity for ponies (Timoney et al. 2000), consistent with its role as an essential virulence factor. Amino acid sequence variation has not been reported in either SzPSe or Se18.9, two other surface/secreted proteins of *S. equi* that elicit strong immune responses during convalescence.

Streptococcus equi enters via the mouth or nose and attaches to cells in the crypts of the oral tonsils and to cilia of the nasopharyngeal tonsils (fig. 4.2). SzPSe may serve as an adhesin during this phase since it has been shown to bind strongly to receptors in crypts of the lingual and palatine tonsils. The aforementioned Fim proteins are also probably involved in the attachment of *S. equi*. The role of host fibronectin in this phase of pathogenesis is unknown. A 40-kDa fibronectin-binding protein, FNEB, expressed on the surface of *S. equi* may contribute to initial attachment. FNZ, a fibronectin-binding protein produced by *S. zooepidemicus*, is also produced by *S. equi* but without a C-terminal anchor, so it may not be functional. Accordingly, *S. equi* has been reported to bind significantly less fibronectin than *S. zooepidemicus*. After a few hours, the organism is difficult to detect on the mucosal surface. Small numbers of organisms are found in epithelial cells in the follicular tissue beneath and in one or more of the lymph nodes that drain the pharyngeal/tonsillar region. By 48 h, clumps of *S. equi* are visible in the lamina propria. Complement-derived chemotactic factors generated after interaction of C1 with bacterial peptidoglycan attract large numbers of PMNs (Mukhtar and Timoney 1988). Failure of PMNs to phagocytose and kill the streptococci appears to be the result of a combination of the hyaluronic acid capsule, putative leukocidal toxin, and the three antiphagocytic and IdeE proteins, SeM, Se18.9, and IdeE. This culminates in the formation of numerous clumps of long chains of extracellular streptococci interspersed among large numbers of degenerating PMNs. Counts of *S. equi* in resulting pus in lymph nodes may exceed 3×10^9 CFU/ml. The enhanced iron acquisition ability of *S. equi* provided by equibactin encoded by ICESe2 is possibly crucial at this stage of pathogenesis since iron is limited in the avascular interior of abscesses. *S. equi* in which *eqb*E was deleted showed a greatly extended generation time (Heather et al. 2008).

Streptolysin S and streptokinase may also contribute to abscess development and lysis by

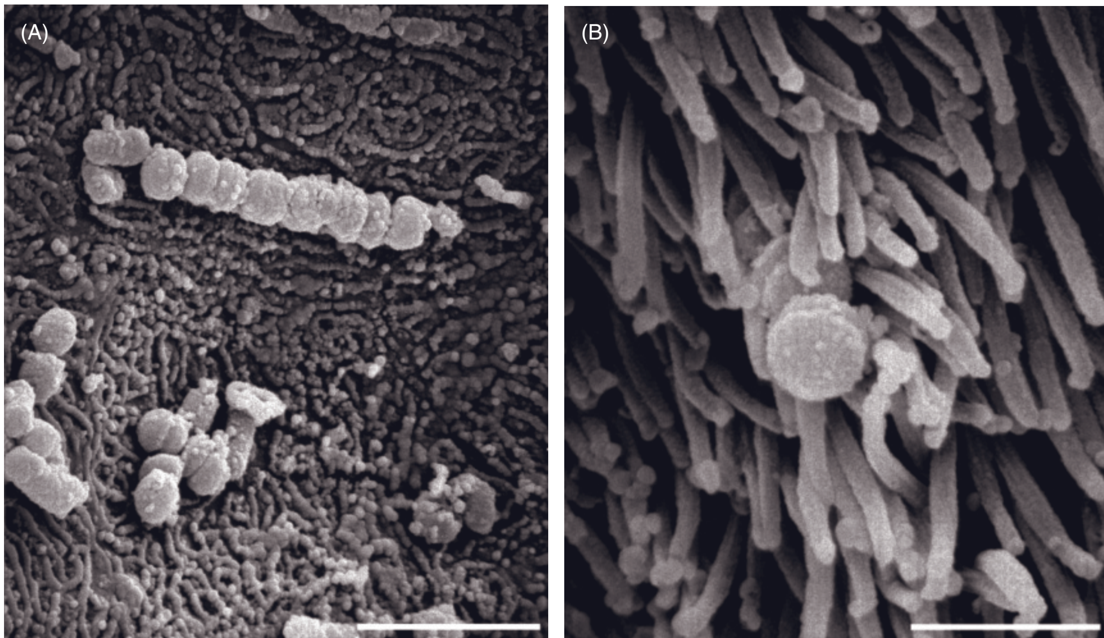

Figure 4.2. Scanning electron micrographs showing attachment of *S. equi* to stratified squamous epithelium of the equine lingual tonsil (A) and to cilia of the columnar epithelium of the nasopharyngeal tonsil (B). 2000×.

damaging cell membranes and activating the proteolytic properties of plasminogen. Although strangles predominantly involves the upper airways and associated lymph nodes, metastasis to other locations may occur. Spread may be hematogenous or via lymphatic channels, which results in abscesses in lymph nodes and other organs of the thorax and abdomen. Metastasis to the brain has also been recorded. Evers (1968) reported bacteremia on days 6–12 in horses inoculated intranasally with *S. equi* and in noninoculated contact horses that became infected. These interesting findings have not been confirmed but clearly show the potential for localization of *S. equi* to body sites other than the lymph nodes of the head and neck and for the formation of circulating immune complexes.

Nasal shedding of *S. equi* usually begins after a latent period of 4–14 days and ceases between 3 and 6 weeks after the acute phase. However, some animals may continue to harbor infection in the guttural pouch months after clinical recovery (Newton et al. 1997).

Immunity

Approximately 75% of horses develop a solid enduring immunity to strangles following recovery from the disease (Todd 1910; Hamlen et al. 1994; Timoney et al. 2007). Horses in the immediate post-convalescent phase are resistant to experimental challenge, with numbers of *S. equi* greatly exceeding those required to produce the original infection (Galán and Timoney 1985). However, about 25% become susceptible to a second attack of the disease within months, which probably represents a failure to produce or maintain an adequate level of the appropriate mucosal and systemic antibodies. Strong serum IgGb responses to proteins, including SeM, Se18.9, SzpSe, Se44.2, Se46.8, Se45.5, and Se42.0, and streptokinase are produced during convalescence. Opsonophagocytic serum IgGb (IgG4) specific for the highly immunogenic SeM appears late in convalescence in some but not in all horses (Timoney and Eggars 1985). In addition, SeM-specific IgGa (IgG1, 2) is induced during and shortly after *S. equi* infection. Strong SeM-specific mucosal

IgA and IgGb responses are seen during the acute and convalescent phases but not following intramuscular vaccination (Sheoran et al. 1997).

Local mucosal IgA responses to *S. equi* require local stimulation and are not correlated with systemic (serum) antibody responses. The proteins that elicit the strongest and most consistent IgA responses during convalescence are H factor-binding Se18.9, fibronectin-binding FNE, and Se46.8, which is of unknown function.

Milk from mares recently recovered from strangles contains IgGb and IgA with specificities similar to those in nasopharyngeal mucus of convalescent horses. Colostral antibodies ingested during the first 24 h of life have also been shown to recirculate to the nasopharyngeal mucosa. Foals that suckle immune mares are usually resistant to *S. equi* infection until weaning.

The antiphagocytic SeM is mouse protective, elicits bactericidal (opsonophagocytic) serum antibody in horses (Timoney and Trachman 1985), and is an important component of commercial strangles vaccines. In addition to SeM, other antigens contribute to protective responses, as shown by an experiment in which a SeM-negative mutant of *S. equi* protected horses against experimental challenge following intranasal vaccination (Timoney et al. 2000). Vaccines that contain either the acid-extracted or enzymatically extracted SeM have conferred only partial protection under conditions of natural exposure. In one study, the clinical attack rate was reduced by 50% following vaccination (Hoffman et al. 1991) under conditions of heavy field challenge.

Substantial bactericidal (opsonic) activity has been noted in sera of vaccinated yearlings that subsequently developed strangles 1 or 2 months later (Timoney and Eggers 1985). This suggests that opsonic activity must be complemented by antibodies to other protective antigens.

A recent attempt to vaccinate horses subcutaneously with pools (1) of recombinant proteins expressed by *S. equi* (SeM, Se44.2, Se75.3, Se42.0, Se110.0, and Se18.9) and (2) adhesin (Fim 1) and other proteins (SzpSe, CNE, Se51.9, Se44.2, and Se46.8) encoded by adjacent loci did not result in enhanced resistance to commingling challenge (Timoney et al. 2007). However, in another study, ponies immunized intramuscularly or intranasally with recombinant EAG (albumin and IgG binding) combined with CNE and subsequently challenged intranasally showed a reduction in nasal discharge and nasal shedding of the challenge strain (Waller et al. 2007).

A successful subunit vaccine must stimulate a blocking immunity that functions at an early stage of infection during and shortly after tonsillar entry. Evidence for this derives from the observation that most horses with acquired immunity following recent recovery from strangles do not make anamnestic serum or mucosal antibody responses to immunoreactive proteins, such as SeM, Se18.9, or SzPSe, following experimental challenge. Thus, acquired immunity must function in the tonsil and possibly involve clearance by macrophages in the follicular-associated epithelium and the lymphoid follicles beneath.

A live nonencapsulated attenuated strain of *S. equi* has been widely used as an intranasal vaccine against strangles in North America since 1998. A progenitor of this vaccine designed to stimulate SeM-specific mucosal and serum responses similar to those of convalescence exhibited a high level of protective efficacy in mice and in experimental ponies (Timoney and Galán 1985). An aro A^- mutant of *S. equi* injected into the inner upper lip has also been shown to generate a short-lived protective immunity. It also is prone to cause purulent reactions at the injection site (Jacobs et al. 2000).

STREPTOCOCCUS ZOOEPIDEMICUS

Although *S. zooepidemicus* shares more than 96% DNA homology with its clonal derivative *S. equi*, it differs greatly in its biology and pathogenicity. Unlike *S. equi*, *S. zooepidemicus* is a mucosal commensal that opportunistically produces disease in situations of virus infection, heat stress, or tissue injury. The specific name "zooepidemicus" derives from its wide host range. Mastitis in cattle and goats; pneumonia, septicemia, and wound infections in lambs, puppies, and greyhounds; septicemias in chickens and dolphins; and lymphadenitis in guinea pigs are examples of its widely ranging pathogenicity. It is the most frequently isolated pathogen from equine joints, lymph nodes, nasal cavities, lungs, and uterus. In situations of concurrent influenza virus infection, high summer temperatures, or transport stress, it can be a devastating and rapidly fatal pathogen in the respiratory tract. It has caused epizootics of peracute, fatal hemorrhagic pneumonia in dog shelters and in greyhound kennels.

Humans infected by exposure to contaminated milk products or by contact with horses are at risk of developing glomerulonephritis and kidney failure.

Virulence Factors

Streptococcus zooepidemicus produces many of the virulence factors listed for *S. equi*. As expected from the greater than 96% DNA homology shared by these species, their protein profiles are similar. Notable differences are absence of genes for known pyrogenic exotoxins, iron binding equibactin E, a functionally antiphagocytic homologue of SeM or Se18.9, as well as homologues of a few other surface-exposed or secreted proteins. Capsule synthesis is highly variable and usually quickly lost following primary culture. Synthesis is tightly regulated, unlike *S. equi*, in which expression is constitutive. Also, many isolates of *S. zooepidemicus* produce hyaluronidase. Isolates from the tonsil and other mucosal sites of healthy animals are almost always unencapsulated. Toxigenic strains have been implicated in outbreaks of septicemia and hemorrhagic pneumonia in greyhounds and shelter dogs but lack genes for the known pyrogenic exotoxins. Three putative fimbriáe-encoding operons have been found in *S. zooepidemicus* MGCS 10565 and two in H70 (Beres et al. 2008). There are also at least three proteins with fibronectin-binding activity, FNZ, FNZ 2, and SFS (Lannergard et al. 2005). Thus, the organism is predicted to have greater attachment/colonizing ability than *S. equi*.

The SzP proteins of *S. zooepidemicus* of equine origin are the basis of the Moore and Bryans typing system (Walker and Timoney 1998) and vary at their N termini and central regions. These proteins are mouse protective, opsonogenic, and found on isolates from different animal hosts. They bind less fibrinogen than the SeM protein of *S. equi*. Variations in the SzP proteins have been useful in establishing the clonal character of equine lung infections and their derivation from the animal's own tonsil (Anzai et al. 2000). As expected, the SzP homologue (SzPSe) in *S. equi* is invariant, consistent with the latter's clonal character. The family of SzP proteins appears to function in the colonization of the nasopharyngeal and genital mucosae as adhesins since they adhere strongly to the lining of tonsillar crypts. The great variation they exhibit in the amino-acid sequence has not been explained but may constitute a mechanism of immune escape.

Immunity

SzP-specific antibodies in equine sera are opsonic (Causey et al. 1995) and mouse protective, and so it is likely that they contribute to the protection in horses and other animals. SzP-specific IgA and IgG in the nasopharyngeal secretions may have a role in controlling the number of *S. zooepidemicus* in the equine tonsil. Also, immunization of mares with bacterial extracts provides some resistance to endometritis caused by *S. zooepidemicus* (Widders et al. 1995). Given the varied antigenicity of *S. zooepidemicus* and the opportunist nature of the infections it causes, vaccines will be difficult to develop. An added concern is the risk of glomerulonephritis associated with complexes of streptococcal proteins such as SzP and specific antibodies (Divers et al. 1992).

STREPTOCOCCUS CANIS

Streptococcus canis belongs to Lancefield group G and is often included with the closely related pyogenic *S. pyogenes*, *S. equi*, and *S. dysgalactiae*. The designation *canis* is reserved for large colony betahemolytic streptococci from animals that react with group G typing antiserum, produce betagalactosidase but not hyaluronidase, and differ in 16Sr RNA sequence. They are thereby distinguished from strains of *S. dysgalactiae* subsp. *equisimilis* of human origin that react with group G antiserum yet are a genetically distinct population. However, animal strains are occasionally isolated from lesions in humans.

The anal mucosa is the main carrier site of *S. canis* in dogs. It may also be found on the genitals, groin, chin, tonsil, mouth, and nose of both dogs and cats. Infections in the hosts are usually sporadic and opportunistic and found in wounds, rectum, mammary gland, prostate, lymph nodes, endocardium, ear canal, uterus, and skin. Outbreaks of abortion, neonatal septicemia, polyarthritis, and infertility in dogs were reported in the late 1930s in England, but not since then, suggesting that the virulent clone involved did not persist. Nevertheless, occasional reports from Europe show that it is still a cause of early mortality in puppies.

A toxic shock syndrome and necrotizing fasciitis associated with *S. canis* was reported in dogs in Ontario in the mid-1990s (Prescott and DeWinter 1997). All of the isolates produced proteases and a CAMP-like factor, but no known pyrogenic

exotoxin or superantigen sequences were found in bacteriophage induced from one of the isolates. Moreover, there was no evidence of clonality that would have supported emergence of a virulent clone. Subsequent studies identified a bacteriophage-inducing effect of fluoroquinolone on an isolate of *S. canis* and the presence of a homologue of pokeweed mitogen on the bacteriophage (Ingrey et al. 2003). Superantigen-like effects of the mitogen would thus explain the ensuing toxic shock and necrotizing fasciitis.

In cats, *S. canis* is the most commonly isolated bacterium from skin and lymph node abscesses, cases of mastitis, conjunctivitis, metritis, and septicemia in kittens (Swindle et al. 1981). Epizootics of contagious streptococcal lymphadenitis have been reported in colonies of laboratory cats and rats (Coming et al. 1991). Fatal infections have been recorded in intensively housed shelter cats (Pesavento et al. 2007).

Virulence Factors

Little has been reported on the virulence factors of *S. canis*. Although hyaluronidase, streptokinase, or hyaluronic acid has not been detected, streptolysin O and M protein are produced in large quantities by recent clinical isolates. The M protein of a virulent *S. canis* clone from an epizootic of neonatal septicemia and mandibular lymphadenitis of juvenile cats has a molecular mass of about 75 kDa. Antiserum against this protein was strongly opsonic for the parent strain and for other clinical isolates of *S. canis* from disease outbreaks. However, isolates from opportunistic infections and from normal healthy cats were phagocytozed equally well by preimmune and M-specific hyperimmune serum, suggesting that an antigenically different protein is present or the protein is present in only low amounts on noninvasive, nonepizootic strains (Timoney and Blanchard 1986).

The epizootic form of feline streptococcosis has been observed mainly in experimental colonies and occasionally in catteries and animal shelters (Blanchard 1987). Persian cats appear to have an enhanced susceptibility. Neonatal kittens are infected from vaginal secretions or from the mouth of the queen when she bites off the umbilical cord close to the abdominal wall. The organism enters the umbilical vein, often causing formation of a small local abscess, and then is carried in the bloodstream to a variety of body sites. Bacterial thrombi form in the liver, spleen, lungs, kidneys, and musculature, resulting in gross and microscopic abscess formation and death within 1 week of birth. The neonatal disease is seen only in litters from young queens. Older queens that have developed antibodies pass these antibodies to their kittens, which then become resistant to infection.

Juvenile streptococcosis occurs in young cats during the postweaning period from 2 to 4 months of age when maternal antibody is waning. *S. canis* acquired earlier during feeding invades the tonsil and local lymphatics of the head and neck, causing a purulent lymphadenitis of the mandibular lymph nodes. Lymphatic obstruction due to infection by *Brugia malayi*, a filarial parasite, predisposes cats to *S. canis* lymphangitis. Lymphostasis apparently compromises bacterial clearance in lymph nodes.

Immunity

Only one M type of *S. canis* has been isolated from outbreaks of feline streptococcosis (Timoney and Blanchard 1986). M-specific opsonic antibodies develop as a result of exposure to *S. canis*, and so older queens tend to have higher opsonic titers and have much lower vaginal colonization rates.

STREPTOCOCCUS PORCINUS

Streptococcus porcinus, of Lancefield group E, U, V, or P, is the cause of a contagious cervical lymphadenitis (porcine strangles) of young 8–10-week-old swine. Although *S. porcinus* has been isolated from a variety of opportunist infections in horses, cats, and humans, pigs are the normal host. As is the case for some of the other pathogenic streptococci, the organism is carried in the tonsils and transmitted by nose contact and via drinking water and feces. Antimicrobial supplementation of feed and changes in swine management have greatly reduced the incidence of porcine strangles in the United States.

Experimental infections result in enlargement of the mandibular, parotid, and retropharyngeal lymph nodes about 2 weeks after infection. Abscessed nodes drain during the following week or become encapsulated.

Virulence Factors

Streptococcus porcinus is encapsulated and produces streptokinase specific for porcine plasminogen (Ellis and Armstrong 1971). An antiphagocytic factor similar to the M proteins of *S. pyogenes* is necessary for virulence (Deynes and Armstrong

1973). Antibody to this protein can be detected by opsonic and long-chain activities. The phagocytic resistance of *S. porcinus* is increased during growth in 10% porcine serum or 2% bovine serum albumin.

STREPTOCOCCUS SUIS

Streptococcus suis is associated with a wide range of clinical syndromes in swine and other domestic animals and is a serious zoonotic pathogen in humans. The principal carrier site in pigs is the palatine tonsil, and transmission is by the respiratory and oral routes. Tonsillar infection may occur shortly after birth and involve any of a range of capsular serotypes. Capsular type 2 is most frequently isolated from cases of septicemia, meningitis, bronchopneumonia, and polyarthritis. Occurrence of disease is stress related and often associated with intensive management.

Tonsillar entry occurs via the crypts in which the organism becomes associated with cells of the myeloid series (macrophages, mature monocytes that are CD163 positive) (Wilson et al. 2007). Innate immune responses involving neutrophils, macrophages, and follicular epithelial cells appear to limit the infection to the tonsil, in which the organism persists.

Bacterial factors that negatively influence innate immunity include suilysin and capsular polysaccharide. Pore-forming suilysin is toxic for neutrophils; capsule reduces phagocytosis by inhibiting their phosphorylation dependent signaling pathways (Segura et al. 2004). Entry of virulent strains into the tonsil is rapid and involves very few organisms, as previously described for *S. equi*. This is followed by bacteremia or septicemia with seeding of joints, meninges, and lungs. The latter may also be directly infected from the upper respiratory tract. However, a sustained high-level bacteremia is necessary for the development of meningoencephalitis. Pneumonia often precedes entry into the CNS. It is unclear whether *S. suis* is attached to or within phagocytes or is free in the plasma during the bacteremic phase. Earlier studies (Williams 1990) indicated that *S. suis* survived in circulating mononuclear cells, which carried it "Trojan horse style" to joints and the meninges. Later, it was shown that carriage also involves surface attachment (Segura and Gottschalk 2002). The choroid plexus is an important portal of entry into the CNS. *S. suis* adheres to and invades endothelial cells of the brain microvasculature. Invasion does not require active bacterial protein synthesis but does require actin microfilaments (Vanier et al. 2004). Damage to these cells by the cytotoxic suilysin may facilitate bacterial translocation into adjacent tissue. In addition, pro-inflammatory cytokine release may result in expression of additional cell adhesion molecules on the endothelial cell surface with increased transmigration of bacteria-laden leukocytes. Hematogenously delivered organisms have been found beneath the surface of brain tissue.

Virulence Factors

Streptococcus suis exists in a multiplicity of phenotypes characterized by the type and presence or absence of capsule and surface-exposed or secreted proteins. This variation, in combination with route of entry and immune status of pigs, determines the virulence potential of a specific isolate (Vecht et al. 1992; Gottschalk et al. 1999). *S. suis* is therefore a highly versatile pathogen that presents an array of virulence factors that appear to function in different combinations to accomplish invasion and lesion production in a variety of conditions. Thus, the presence or absence of a specific virulence factor does not necessarily establish a condition of virulence or avirulence. The availability of genome sequence of *S. suis* has greatly expanded the list of putative virulence factors, many of which are anchored to the surface or are secreted.

The best studied virulence-associated factors are the capsular polysaccharide, the muramidase-released protein (MRP), the extracellular protein factor (EF), and the hemolytic and cytotoxic suilysin. Other less studied factors include serum opacity factor (OFS), fibronectin, and plasminogen-binding enolase (SsEno), surface antigen one (SAO), proteins that perform D-alanylation of lipoteichoic acid (LTA), proteases, adhesins including fibrinogen binding protein (FBPS), and a zinc-binding lipoprotein.

Capsular Polysaccharide

The antiphagocytic sialic acid-rich capsular polysaccharide may function by blocking deposition of C3 and activation of the alternative complement pathway. It may also aid in intracellular survival in phagocytes and in adhesion. A polymer of rhamnose, glucose, galactose, N-acetylglucosamine, and sialic acid, capsular polysaccharide exists in at least 35 different serologic variants, the most frequent being types 2, 3, 1/2, 8, and 4. The distribution of serotypes varies geographically and temporally. For

example, capsular type 2 is more prevalent in Europe than in North America. Antibodies to capsular types are opsonic and partially protective and are elicited at low levels during convalescence. Higher levels of antibodies are associated with greater protection.

Suilysin

The 64-kDa suilysin is a thiol-activated hemolysin with homology to pneumolysin, streptolysin O, and listeriosin. This toxin family produces transmembrane pores in target cells. Suilysin is thermolable and either secreted or loosely cell bound. Forty-four percent of isolates from the lung express the toxin compared with 80–90% of isolates from other sites in pigs (Staats et al. 1999). Thus, the toxin is not an essential virulence factor.

Muramidase-Released Protein

Muramidase-released protein is a 136-kDa surface protein of unknown function with a typical gram-positive cell wall anchor sequence (Smith et al. 1992). A proline-rich region adjacent to the C terminus is followed by an extensive series of repeats, the number of which is positively correlated with virulence. Deletion of the gene does not result in loss of virulence for newborn piglets (Smith et al. 1996), although specific antibody contributes to protection against strains expressing the protein. Variants of MRP have been described.

Extracellular Protein

Extracellular protein factor and MRP are positively correlated with virulence but are not essential virulence factors. EF is a 110-kDa secreted protein of unknown function that is often associated with pathogenic strains of *S. suis*. Variants of EF found in nonpathogenic isolates have several 76 amino acid repeats at their C termini.

North American isolates of *S. suis* type 2 that express EF, MRP, and suilysin are usually highly virulent and may be clonal.

Outbreaks of human disease in China in recent years have been caused by a specific tetracycline-resistant clone of *S. suis* that became widespread in local swine populations. This clone had acquired the transposable element Tn*916* together with genes for MRP, suilysin, EF protein, and a salivaricin-regulated two-component signal transduction system (SalK/SalR; Ye et al. 2008). This highly virulent clone caused numerous fatalities in humans.

Adhesins and Other Virulence-Associated Factors

An 18-kDa peptide of *S. suis* that binds to the disaccharide galactosyl-(alpha 1-4)-galactose sequence on cell surface glycolipids is found on most isolates. Paradoxically, the adhesin is covered by the capsule, which therefore interferes with its activity. It is highly immunogenic and induces bactericidal antibody in mice. GADPH is also involved in adhesion to host cells.

Immunity

The self-limiting nature of disease in contained pig populations strongly implicates the emergence of an acquired, protective immune response. Repeated inoculation of pigs with live and formalin-killed cultures of *S. suis* type 2 results in a strong protective response effective against homologous challenge and characterized by the appearance of both serum IgM and IgG specific for surface components (Holt et al. 1988). Immune sera mediate killing of *S. suis in vitro*. A 94-kDa antigen band, reactive with protective pig sera, stimulates bactericidal and mouse protective antibodies and may be part of the cell envelope 135-kDa protein that binds fibronectin (Smith et al. 1992).

Subunit vaccines prepared from suilysin with MRP and EF have protective efficacy, albeit limited to challenge with homologous strains that express these factors (Jacobs et al. 1996). A 46.4-kDa protein of unknown function has also been shown to stimulate protection in pigs challenged with a homologous serotype (Okwumabua and Chinnapapakkagari 2005).

Capsular polysaccharide, a T-independent antigen, induces only a weak antibody response specific for the serotype but is potentially an important protective immunogen (Wisselink et al. 2002). Opsonic responses are improved by the addition of Freund's incomplete adjuvant. Killed cells of a wild encapsulated strain stimulated protection that reduced morbidity and mortality, whereas a similar vaccine prepared from an isogenic nonencapsulated mutant protected only against mortality. Another protein vaccine candidate, Sa0 (110 kDa), has shown promise as an opsonogenic, protective immunogen in pigs when formulated with Quil A (Li et al. 2007). The success of this vaccine suggests that a Th1 response contributes to protection.

Although there is evidence that favors the inclusion of MRP and EP in subunit vaccines combined

with the common capsular antigens, neither of these proteins has been shown to induce protection. In addition, capsular polysaccharides or their specific epitopes should be conjugated to a suitable carrier protein to elicit a strong T cell response and a high level of antibody.

The lack of efficacy of formalin-killed vaccines under conditions of heterologous challenge is possibly explained by lack of essential protective immunogens either because their amounts are minute on cultured cells or because denaturation of protective epitopes occurs during vaccine preparation.

STREPTOCOCCUS PNEUMONIAE

Although a common and serious pathogen of humans, the highly host-adapted *S. pneumoniae* is seldom isolated from clinical disease in animals. However, a unique clone of capsular serotype 3 has been associated with lower airway disease in horses in combination with other bacteria and respiratory viruses (Mackintosh et al. 1988, Chapman et al. 2000). A case of pneumonia in a neonatal foal has also been reported (Meyer et al. 1992). Equine isolates of *S. pneumoniae* are remarkable because they exhibit deletions in the *lytA* and *ply* genes for the virulence factors autolysin and pneumolysin and are genetically almost identical to each other. Moreover, they are genetically distinct from isolates of *S. pneumoniae* serotype 3 from humans (Whatmore et al. 1999). Experimental intratracheal inoculation of ponies was followed by fever, cough, ocular and nasal discharge, and lesions of lobar pneumonia (Blunden et al. 1994).

Virulence Factors

The well-studied virulence factors of *S. pneumoniae* of human origin include the capsular polysaccharide, pneumolysin, autolysin, neuraminidase, hyaluronidase, cell wall peptidoglycan, teichoic acid, and phosphorylcholine. A large number of surface-anchored proteins are also expressed, including ZmpB, a zinc metalloprotease involved in processing and export of other proteins, an IgA protease, peptide permeases AmiA and PlpA, neuraminidases NanA and NanB, glycolytic enzymes, a fibronectin-binding enolase, and an array of 12 choline-binding proteins, including PspA (a protective antigen), LytA (an autolysin), and CppA (an adhesin). A notable feature of the cell wall surface is the presence of free choline and choline covalently linked to teichoic and lipoteichoic acids. Proteins with choline-binding repeats attach to these cholines on the bacterial surface.

In addition to virulence molecules unique to pathogens, proteins involved in carbohydrate catabolism in pathogenic and nonpathogenic bacteria may also contribute to virulence. Inactivation of genes for these proteins results in reduced virulence in *S. pneumoniae* and *S. agalactiae* (Shelburne et al. 2008).

Adhesion of *S. pneumoniae* to epithelium of the tonsil and soft palate of ponies has been noted following experimental infection (Blunden et al. 1994). Invasion triggers a number of host responses, including the coagulation cascade with thrombus formation, the complement cascade with accumulation of leukocytes, and the chemokine/cytokine cascade that ultimately leads to increased vascular permeability and leukocyte recruitment. Resistance to phagocytosis is mediated by a complex polysaccharide capsule that forms a hydrophilic gel on the surface of the organism. This gel shields the bacterium from antibodies and complement proteins. In addition, capsular sialic acid contributes to the antiphagocytic effect by inhibiting complement amplification and alternative pathway activation. Intrinsic complement inactivation mechanisms, which degrade C3b bound to the bacterial surface and prevent further C3 deposition, are also facilitated by capsular sialic acid. Capsular material has, however, been noted in the alveolar macrophages of ponies experimentally infected with *S. pneumoniae*, indicating that successful phagocytosis does take place. It is unclear how this relates to the clinically mild self-limiting nature of the naturally occurring respiratory disease of young horses. Alveolar necrosis has also been observed in experimentally produced lesions in ponies (Blunden et al. 1994).

Increased numbers of *S. pneumoniae* are associated with the stress of race training and with lower respiratory tract inflammatory disease, suggesting that the host/parasite interaction is opportunistic. Increased respiration during intense exercise may result in aspiration of *S. pneumoniae* from the tonsil and soft palate. At the same time, impairment of the mucociliary escalator mechanism and fluid accumulation may contribute to failure to clear aspirated organisms. Bacteria, including *S. zooepidemicus*, that proliferate in the highly cellular exudate generate highly inflammatory streptococcal cell wall products.

Immunity

Much of the information on protective immunity of *S. pneumoniae* is based on experiments in mice and may not be relevant to acquired protective immunity in horses. Moreover, studies on the immunogenicity of type 3 capsule for horses have not been reported. In humans, type-specific capsular antibody produced during convalescence is opsonizing and protective for mice. However, capsular polysaccharide is often a weak immunogen. Using the genome sequence of *S. pneumoniae*, Wizeman et al. (2001) identified a set of surface and secreted proteins, reactive with convalescent human serum and protective for mice. These include Psp A, Psp C, pneumolysin, Psa A, antolysin, and neuraminidase A and B. A vaccine composed of a combination of a reduced set of these proteins would have major advantages over a polysaccharide-based product in that it would not be seroptype specific and T cell dependent and would elicit a strong immune response.

Another approach to effective immunization is the use of intranasally administered attenuated *S. pneumoniae* in which virulence genes have been deleted (Roche et al. 2007). This has resulted in serotype-independent systemic and mucosal protection in mice and resembles that induced previously by attenuated *S. equi* and *S. porcinus* in horses and pigs, respectively.

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Comparisons of genome sequences have confirmed that the breadth and diversity of host and tissue tropisms of the pathogenic veterinary streptococci resulted from multiple paths of evolution arising from acquisition of new genes, modifications or loss of existing genes, and changes in regulatory networks. These genetic events resulted in phenotypes uniquely fitted for invasion of specific tissues, such as the equine tonsil (*S. equi*), ruminant mammary gland (*S. agalactiae* and *S. uberis*), and pig tonsil (*S. suis*). In contrast, other successful and closely related streptococcal pathogens, including *S. zooepidemicus* and *S. dysgalactiae* subsp. *equisimilis*, exhibit almost no host adaptation and are capable of causing disease only as opportunists in situations of preexisting damage to mucosal barriers or impaired immune defenses.

Streptococcal molecules and structures involved in host entry, evasion of innate immune responses, and subsequent multiplication are of great interest and so too are the cells and fixed and soluble factors of the animal host that participate in or facilitate initial invasion. Past research efforts, heavily invested in streptococcal proteins that bind promiscuously to abundant host molecules, such as fibronectin, immunoglobulins, and fibrinogen, have not been helpful in understanding host specificity. Hopefully, the more comprehensive set of surface and secreted proteins identifiable by analysis of the genomic sequences of *S. equi*, *S. zooepidemicus*, *S. agalactiae*, *S. uberis*, *S. pneumoniae*, and *S. suis* has the potential to advance this goal. However, a significant hurdle to be overcome is that many of the proteins predicted from the genomic sequence of each pathogenic streptococcus are novel and of unknown function. Some are not expressed. or function as virulence factors only in a specific sequence conformation such as for SeM and SzM of *S. equi* and *S. zooepidemicus*, respectively. Unlike SeM, SzM does not have antiphagocytic activity. Other proteins may require proteolytic cleavage or other modification to produce a form of the molecule active in pathogenesis.

Immunization-challenge studies with proteins of *S. pneumoniae* as an example indicate that multiple proteins are required for optimum virulence, pathogenicity, and protective immunity. Elucidation of effective combinations can only be discovered by experiment in the natural host since there is abundant evidence showing that results of immunization experiments in mice are frequently not transferable.

Experiments in the natural host using different combinations of a wild strain and its mutants deleted for each putative virulence factor gene are therefore necessary to establish which proteins actually contribute to pathogenesis and at what stage. This entails screening tissues of animals killed a short time after infection for the presence of each mutant to establish how many and how far each mutant penetrated. Clearly, such experiments are more feasible in pigs than in large and expensive hosts, such as cows and horses. An alternative approach, using the natural host, will be the use of microarrays of sequences of defined sets of genes for exposed and secreted proteins to hybridize to labeled RNA from the host at different stages of infection to identify proteins expressed at these times. Another approach that has proved valuable in identifying proteins with

possible virulence roles has been to screen expression gene libraries with pools of convalescent IgG and mucosal IgA. An advantage of this approach is that it identifies proteins expressed only *in vivo*.

Recent technological advances combined with genomic sequence analysis are grounds for optimism that improved understanding of and access to the streptococcal components with potential to contribute to the pathogenesis of animal disease will provide an experimental framework for the development of more effective therapeutic and vaccine modalities. However, this optimism must be tempered by early results that reveal an unexpectedly large number of potential targets that vary with pathogen and host and which will require extensive animal experimentation to unravel.

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5
Staphylococcus

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INTRODUCTION

Staphylococci are gram-positive cocci (0.5–1.5 µm in diameter) that occur singly, in pairs, tetrads, short chains (three or four cells), and irregular grape-like clusters. More than 50 species and subspecies are currently known, and new taxons and names continue to be described.

CHARACTERISTICS OF THE ORGANISM

Recently, the taxonomy of the phylum *Firmicutes*, to which the staphylococci belong, has been completely revised (Ludwig et al. 2009). The genus *Staphylococcus* belongs to the class *Bacilli*, order *Bacillales*, family *Staphylococcaceae*, together with the genera *Macrococcus*, *Jeotgalicoccus*, and *Salinicoccus*. The minimal standards for assigning an organism to the genus *Staphylococcus* include genotypic as well as phenotypic criteria (Freney et al. 1999).

Genotypic Criteria

The DNA of staphylococci has a guanine plus cytosine (G + C) content of 30–39 mol%. Other genotypic criteria to assign an unknown species to the genus *Staphylococcus* are based on phylogenetic trees constructed by comparison of 16S rRNA or 23S rRNA sequences.

The staphylococci can be divided by 16S rRNA gene sequence analysis (Takahashi et al. 1999) and whole-genome DNA–DNA reassociation studies into several phylogenetic species groups. Species within these groups typically share important characteristics, are usually difficult to differentiate from each other, and may occupy similar ecological niches or show similar pathogenicity potentials.

To some degree, the staphylococci have evolved along with their host species. Different animal species have different staphylococcal microflora. This can be seen at the species level and at the strain level. At the infraspecies level, genotypic studies have characterized staphylococcal species in epidemiological (fingerprinting) studies at different levels up to the strain level. From the veterinary and zoonotic perspectives, typing methods distinguishing between host-associated groups of strains (ecovars) can be useful. However, the host association is not absolute since it has been shown that certain clonal strains can colonize different animal species, as is, for instance, the case for the so-called animal-associated methicillin-resistant *Staphylococcus aureus* (MRSA), which will be discussed further on.

Phenotypic Criteria

The catalase reaction is positive for all *Staphylococcus* species, except for *S. aureus* subsp. *anaerobius* and *Staphlococcus saccharolyticus*. Staphylococci are facultative anaerobic bacteria, except for *S. aureus* subsp. *anaerobius* and *S. saccharolyticus*, which are strictly anaerobic. The ultrastructure and chemical composition of the cell wall of staphylococci are those as typical for gram-positive bacteria (Beveridge 2000). The wall is made up of peptidoglycans, teichoic acid, and proteins (Schleifer et al. 1976).

On the basis of the coagulase test, the genus *Staphylococcus* was originally divided into the

coagulase-positive species *S. aureus* and coagulase-negative staphylococci (CNSs). In humans, staphylococci are among the most common causes of bacterial infections, and *S. aureus* is the most important pathogenic *Staphylococcus* species. In veterinary medicine, it used to be stated that three staphylococcal species were of major pathogenic importance, namely, *S. aureus*, *Staphylococcus hyicus*, and *Staphylococcus intermedius* (Devriese 1990). This statement stays correct for *S. aureus* and *S. hyicus*, but following the description of a new species, *Staphylococcus pseudintermedius* (Devriese et al. 2005), it appears that strains formerly identified as *S. intermedius* should be reclassified (Bannoehr et al. 2007; Sasaki et al. 2007). All canine and some equine staphylococci previously identified as *S. intermedius* are now considered to be *S. pseudintermedius*. Other previous *S. intermedius* strains (from, e.g., domestic pigeons and horses) appear to belong to the species *Staphylococcus delphini*. Real *S. intermedius* strains seem to be confined to feral pigeons. Phenotypic tests offer some help in the differentiation of *S. intermedius*, *S. pseudintermedius*, and *S. delphini* (Sasaki et al. 2007). Unlike the other two species, *S. intermedius* acidifies β-gentiobiose and D-mannitol but fails to react in arginine dihydrolase tests. Phenotypic distinction between *S. delphini* and *S. pseudintermedius* is difficult. Non-canine isolates are probably better reported as strains belonging to the *S. intermedius* species group (SIG; Takahashi et al. 1999).

Staphylococcus aureus and *S. intermedius*, *S. pseudintermedius*, and *S. delphini* are coagulase positive, while coagulase production in *S. hyicus* is variable but mainly negative or weak (Kloos and Schleifer 1986). Two additional *Staphylococcus* species have the ability to produce coagulase: *Staphylococcus lutrae* and *Staphylococcus schleiferi*. *S. lutrae* has been isolated from otters (Foster et al. 1997). *S. schleiferi* has long been regarded as a coagulase-negative species until, in 1990, a new subspecies was described from otitis externa from dogs, namely *S. schleiferi* subsp. *coagulans*, which is able to produce coagulase (Igimi et al. 1990). The other subspecies, *S. schleiferi* subsp. *schleiferi*, does not clot rabbit plasma. All these species, except *S. aureus*, belong to a phylogenetically closely related *S. intermedius—S. hyicus* species group.

Because of this complex situation, it is more convenient to use the terms "major" and "minor" pathogenic staphylococci than coagulase-positive and coagulase-negative staphylococci. This terminology has been adopted for many years in mastitis bacteriology. The major pathogenic species are *S. aureus*, *S. hyicus*, and members of the SIG, while all others are to be considered minor pathogens. In veterinary medicine, the latter are mainly of importance in subclinical mastitis, while the former play a role in a multitude of well-recognizable pathological conditions in many different animal species. However, the minor pathogenic staphylococci can be involved in severe disease affecting debilitated patients in hospital conditions. To date, this has only been well documented in human hospitals, but it may become more common as complex surgery and care of aged patients become more common in companion animal medicine.

PATHOGENIC *STAPHYLOCOCCUS* SPECIES

Major Pathogenic *Staphylococcus* Species

In humans, *S. aureus* is a well-documented opportunistic pathogen. It may cause skin infections, as well as enterotoxemia and septicemic infections, and is furthermore important as the cause of toxic shock syndrome (TSS). *S. aureus* infections may be community acquired but are also important as nosocomial infections. An important impediment in the control of *S. aureus* infections is antimicrobial resistance. MRSAs are a major clinical and epidemiological problem in human hospitals. MRSAs have a tendency to accumulate, in addition to their methicillin-resistance gene, additional unrelated resistance determinants in their genome. This has led to the evolution of MRSA strains resistant to almost all commonly used antimicrobial agents. From the original description of MRSA in animals (Devriese and Hommez 1975) to the end of the 20th century, only MRSA strains showing characteristics commonly seen in human epidemic strains were isolated. Animals apparently were infected by their human attendants. More recently, a so-called animal-associated MRSA strain has emerged in different animal species. This strain, characterized with multilocus sequence typing (MLST) to belong to the clonal complex ST 398, was first isolated in pig farms (Voss et al. 2005) but has also been revealed in poultry (Nemati et al. 2008a) and horses (Van den Eede et al. 2009) and appears to be present throughout Europe. This clone is often transmitted from

animals to humans and has important zoonotic significance.

Typical animal strains of *S. aureus* are important pathogens in veterinary medicine, causing disease in cattle, small ruminants, poultry, rabbits, pigs, and horses (Devriese 1990) and in many other animal species as well. In cattle and small ruminants, *S. aureus* is one of the major causes of mastitis. Joint infections, osteomyelitis, and septicemia due to *S. aureus* are described in poultry (McNamee and Smyth 2000). In rabbits, *S. aureus* mainly causes mastitis, pustular dermatitis, subcutaneous abscesses, and pododermatitis (Okerman et al. 1984). In horses, the bacterium may cause dermatitis and cellulitis (Devriese 1984). Pigs may sporadically suffer from septicemia due to a *S. aureus* infection (Devriese 1990). The ST 398 MRSA strain has also been described as a cause of exudative epidermitis in pigs (van Duijkeren et al. 2007).

Staphylococcus pseudintermedius and the other species of the SIG are the most important non-*aureus* species. *S. pseudintermedius* is the predominant coagulase-positive *Staphylococcus* causing skin infections in dogs (Bannoehr et al. 2007). Species from the SIG may also cause septicemia in ducks and pigeons and dermatitis in mink and horses (Devriese 1990; Hesselbarth and Schwarz 1995).

Exudative epidermitis (greasy pig disease) and sporadic joint infections or cystitis in pigs are mostly caused by *S. hyicus* (Wegener and Skov-Jensen 2006). This species appears to be involved also in flank biting and ear-tip necrosis syndrome (Mirt 1999). *S. hyicus* plays an important role in exudative skin infections of cattle (secondary to mange), horses, and even poultry (secondary to pox lesions).

Nearly all phenotypic studies at the infraspecies level have been carried out with *S. aureus* strains. Phage typing has been extraordinarily useful in epidemiologically important characterizations of human strains and continues to be so, supplemented by molecular fingerprinting methods. Phage typing has been used with animal strains of *S. aureus* and with other staphylococcal species, but the methods are labor intensive and phage supplies are difficult to standardize. Therefore, molecular methods are probably better suited for veterinary strains. Up until now, pulsed field gel electrophoresis (PFGE) remains the standard procedure for these molecular methods. However, *spa* typing and MLST are also applied quite regularly for animal strains, especially in the case of MRSA.

Minor Pathogenic *Staphylococcus* Species

The other species, which are mainly coagulase negative, have long been regarded as nonpathogenic, but in recent years their important role as pathogens and their increasing incidence in human infections have been demonstrated (Huebner and Goldmann 1999). They are widespread on human and animal body surfaces and constitute a major component of normal skin and mucosal microflora. Specific virulence factors are not as clearly established as they are in *S. aureus*. The species most frequently described in humans is *S. epidermidis* (Frebourg et al. 2000).

In veterinary medicine, these species have been most extensively studied in relation to bovine mastitis. They are regarded as potential primary udder pathogens (Jarp 1991), but they also protect to some extent against infections with other udder pathogens, such as *S. aureus* (Lam et al. 1997; De Vliegher et al. 2004). Species described in subclinical as well as clinical mastitis of dairy cattle are *Staphylococcus simulans*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Staphylococcus warneri*, *Staphylococcus hominis*, *Staphylococcus epidermidis*, *Staphylococcus sciuri*, *Staphylococcus cohnii*, *Staphylococcus capitis*, *S. hyicus*, and *Staphylococcus caprae* (Jarp 1991; Devriese et al. 1994; Santos et al. 2008). There is a negligible difference in pathogenicity between these species (Jarp 1991). CNSs isolated from milk samples of mastitic small ruminants belong to the following species: *S. epidermidis*, *S. simulans, S. xylosus*, *S. chromogenes*, *S. hyicus*, *S. sciuri*, *S. warneri*, *S. haemolyticus*, *S. hominis*, *S. caprae*, *Staphylococcus lentus*, *Staphylococcus equorum*, *S. capitis*, *Staphylococcus arlettae*, *Staphylococcus saprophyticus, Staphylococcus saccharolyticus*, and *Staphylococcus lugdunensis* (Deinhofer and Pernthaner 1993; Fthenakis et al. 1994; da Silva et al. 2004). These identifications should be viewed with caution since different methods of species identification may not yield the same results (Burriel and Scott 1998; Sampimon et al. 2009).

SOURCES OF THE BACTERIUM

Epidemiological studies have revealed several bacterial sources and carrier sites varying according to the animal species. The staphylococci are intimately associated with animals and cannot be regarded as environmental bacteria.

Ruminants carry staphylococcal strains on their skin, which includes the teat skin. The species distribution, however, differs in different body regions, and the teat skin and teat apex microflora differs from the microflora associated with hairy skin. The development of mastitis is related to the entrance in the teat duct of staphylococci colonizing the teat apex.

In poultry, *S. aureus* has been implicated in a variety of infections, but it has also been isolated from the skin and nares of apparently healthy chickens, from healthy slaughtered poultry, from chick fluff in hatcheries, and from the air in poultry houses (Butterworth 1999). The strains isolated from cases of staphylococcosis are similar to those present on the skin and in the nares of apparently normal birds in poultry flocks. Two or more strains can coexist in the same flock, although the principle of bacterial interference, by which one strain can inhibit colonization by another strain, has also been described in poultry (Devriese 1981). The intimate association of the so-called poultry ecovar of *S. aureus* with poultry suggests that the animals acquire their *S. aureus* populations from other chickens rather than from environmental sources. *S. aureus* strains found on day-old chickens may originate from the parent flocks (Devriese 1981). MRSA ST 398 has been recovered from the nose and cloaca of healthy chickens. This MRSA clone was first and most extensively described in pigs, but it is not clear whether direct or indirect contact with pigs is necessary for the birds to become carriers.

Rabbits may be carriers of *S. aureus* strains on several body sites (Hermans et al. 1999). Transmission from humans to rabbits or between rabbits may be direct or indirect, through cage materials, flying hairs, and food chains. Direct transmission of *S. aureus* bacteria may be between does and suckling young, between litter mates, and between stable mates (Devriese et al. 1981; Matthes 1995). Often, relationships exist between rabbitries infected with identical *S. aureus* strains, and intake of new breeding rabbits in the flock is probably the most important source of infection. Sperm (even after artificial insemination) also constitutes a potential source of infection by highly virulent *S. aureus* strains in rabbits (Rossi et al. 1995).

In young pigs, *S. hyicus* is the causative agent of exudative epidermitis, but it also occurs frequently on the skin, in the nasal cavities, or in the external ears of healthy pigs (Takeuchi et al. 1985). Healthy breeding sows may transmit the bacterium from their vaginal microflora to the skin of their offspring during birth. The vaginal strains of the sow thus become part of the skin microflora of the piglets (Wegener and Skov-Jensen 1992). MRSA strains belonging to the clonal complex ST 398 can be present in the nares and on the perineum of pigs (Voss et al. 2005; Guardabassi et al. 2007).

Dogs may be carriers of *S. pseudintermedius* (Bannoehr et al. 2007) in the nares, in the oropharynx, and on the anal ring (Harvey and Noble 1998). The same strains may be found in the nares of the dog owner (Harvey et al. 1994).

BACTERIAL VIRULENCE FACTORS

Several potential virulence factors have been described as important in staphylococcal infections. Most of these factors have been studied in *S. aureus*, but some of them have also been found in other *Staphylococcus* species. The virulence factors can be divided into cell-associated components, exoenzymes, and exotoxins. In response to changing host environments, *S. aureus* can switch on selected sets of genes to enhance its chances for survival. Regulation of the production of virulence factors in response to cell density, energy availability, and environmental signals is achieved by a complex regulating system (two-component systems and transcription factors). The most important and well-described two-component system (or signal receptor) is encoded by the accessory gene regulator (*agr*). The latter also plays a role in quorum sensing (i.e., the ability of the bacteria to communicate with each other via diffusible signal molecules and to regulate their genes in concert with cell population density). In-depth overviews of the regulatory systems of staphylococcal virulence have been written by Novick (2003) and Cheung et al. (2004).

The known virulence factors of staphylococci (mainly *S. aureus*) will be described in the following paragraphs.

Cell-Associated Components

Protein A

Protein A is a surface protein of *S. aureus* that binds IgG molecules by their Fc region. In serum, the bacteria bind IgG molecules in the wrong orientation, which disrupts opsonization and phagocytosis (Grov 1973).

Capsular Polysaccharide

Capsular polysaccharides, representing 11 serologically distinct capsule types, have been identified in *S. aureus* isolates of humans and cattle. These capsular polysaccharides have been proposed to interfere with host defense mechanisms by inhibiting the attachment of antibodies. They have also been described to bind to epithelial and endothelial cells and to monocytes, and they induce the release of cytokines (Soell et al. 1995).

Peptidoglycan and Lipoteichoic Acid

It has been suggested that the cell wall components peptidoglycan and lipoteichoic acid function as virulence factors and probably stimulate cytokine release. Peptidoglycan and lipoteichoic acid seem to act in concert to produce shock, while neither purified lipoteichoic acid nor peptidoglycan individually produced shock in a rat model (Projan and Novick 1997).

Adhesins

Staphylococcal bacteria may express proteins on their surface that promote attachment to host proteins, such as fibronectin, laminin, vitronectin, and collagen, that form the extracellular matrix of epithelial and endothelial surfaces (Mamo et al. 1988; Gillaspy et al. 1998). In addition, a number of strains express a fibrinogen-binding protein (clumping factor), which is responsible for attachment to blood clots and traumatized tissue. Interaction with collagen may also be important in promoting bacterial attachment to damaged tissue where the underlying layers have been exposed. Indications that staphylococcal matrix-binding proteins are virulence factors have come from studying defective mutants in adherence experiments and from binding blocking assays. Besides the role that these matrix-binding proteins or so-called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) play in tissue binding, they are also suggested to be responsible for the formation of biofilms. A biofilm is "a microbial derived sessile community characterized by cells that are irreversibly attached to a substratum of interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced, and exhibiting an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton 2002, p. 168). Biofilm formation has also been indicated to occur in bovine mastitis (Vasudevan et al. 2003).

Exoenzymes

Coagulase

Coagulase is an extracellular protein that binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity is activated in the complex, leading to conversion of fibrinogen to fibrin. Probably, the bacteria protect themselves from phagocytic and immune defenses by causing localized clotting (Projan and Novick 1997).

Lipase

In response to an infection, the host can produce a variety of fatty acids and other lipid molecules acting as surfactants that disrupt the bacterial membrane, especially when an abscess is formed. Lipases and an enzyme known as fatty acid-metabolizing enzyme (FAME) produced by *S. aureus* are proposed to have a negative effect on immune function. Furthermore, one of the roles of lipase is probably also to release nutrients from the environment (Projan and Novick 1997).

Hyaluronate Lyase and Hyaluronidase

Hyaluronidase and hyaluronate lyase represent a family of enzymes that digest hyaluronic acid and are associated with virulence. It has been suggested that depolymerization of hyaluronic acid present in connective tissue contributes to the infective process by promoting spread through degradation of tissues (Farrell et al. 1995).

Proteases

The best described staphylococcal protease is a serine protease, known as the V8 protease. Proteases have been proposed to function in blocking the action of antibodies by cleaving and inactivating them since the V8 protease has the ability to cleave and inactivate IgG antibodies *in vitro*. A second role for proteases may involve protection against antimicrobial peptides, such as the neutrophil defensins or the platelet microbiocidal proteins. These proteases may contribute to the destruction of tissue proteins and enhance invasiveness. The V8 protease is responsible for the degradation of fibronectin-binding proteins, thus inducing bacterial spread after the initial adherence step. Another possible role for these proteases is obtaining nutrients from the environment (McGavin et al. 1997).

Exotoxins

Enterotoxins and Toxic Shock Syndrome Toxin

Staphylococcus aureus secretes enterotoxins/enterotoxin-like toxins and TSS toxin (TSST-1), which have or may have superantigen (SAg) activity. SAgs stimulate T cells nonspecifically without normal antigenic recognition. They directly cross-link conserved regions of the variable domains of the T cell receptor β-chain with major histocompatibility complex (MHC) class II molecules on antigen presenting cells. This results in a strong stimulation of T cells, which respond with proliferation and massive cytokine release. In this way, SAgs activate up to 25% of all T cells. In contrast, conventional antigens only stimulate around 0.001% of T cells. The fact that cytokines are released in large amounts causes the symptoms of TSS (Fraser et al. 2000; Grumann et al. 2008).

Thus far, nine enterotoxins (SEA-SEE and SEG-SEJ) and nine enterotoxin-like toxins (SElK-SElR and SElU) have been identified in *S. aureus*. Diseases due to *S. aureus* in which these toxins play a predominant role have mainly been described in humans but have also been seen in cattle, goats, and sheep (Ho et al. 1989). In dogs, enterotoxins produced by *S. pseudintermedius* have been described, and a canine type C staphylococcal enterotoxin (SEC canine) has been characterized molecularly, biologically, and immunologically (Edwards et al. 1997). Enterotoxins cause diarrhea and vomiting when ingested and are responsible for staphylococcal food poisoning (Bergdoll 1983). TSST-1 is released into the bloodstream and is the cause of TSS. Enterotoxins can also cause TSS if they enter the circulation. TSS can occur as a sequel to any staphylococcal infection if an enterotoxin or TSST-1 is released systemically and the host lacks appropriate neutralizing antibodies (Bohach and Foster 2000). Studies dealing with enterotoxins in CNS of animal origin are quite rare (Nemati et al. 2008b). Valle et al. (1990, 1991) found production of SEA, SEB, SEC, and SEE in CNS strains isolated from goat's milk and suggested that goats are an important reservoir of enterotoxigenic staphylococci. Orden et al. (1992) found that two *S. xylosus* strains out of 40 CNS strains isolated from bovine mastitis were enterotoxigenic, producing SEC.

Epidermolytic Toxins

The exfoliative toxins ETA and ETB, produced by *S. aureus*, cause a spectrum of disease ranging from bullous impetigo to the scalded skin syndrome in humans, which results in widespread blistering and loss of the epidermis. The toxins have a specific esterase activity, but it is not clear how this causes epidermal splitting. There is some evidence that the toxins have protease activity, so it is also possible that they target a specific protein involved in maintaining the integrity of the epidermis (Noble and Lloyd 1997). ETC has been described in *S. aureus* from a horse with skin infection (Noble and Lloyd 1997).

Exudative epidermitis in pigs caused by virulent *S. hyicus* strains is due to toxins that resemble *S. aureus* exfoliative toxins in their activity but are not closely related immunologically (Tanabe et al. 1993). The exfoliative toxins of *S. aureus* and *S. hyicus* also have different species specificities. ETA and ETB affect the skin of humans and mice but not pigs, whereas the exfoliative toxin of *S. hyicus* affects pigs and chickens but not mice (Wegener and Skov-Jensen 2006). In *S. pseudintermedius* strains from dogs, an exfoliative toxin-like toxin has been described, proposed by the authors to be a novel type of ET, *S. intermedius* exfoliative toxin (SIET) (Terauchi et al. 2003).

Hemolysins (α, β, γ, δ) and Leukocidin

Alpha-toxin is the best-characterized and most potent membrane-damaging toxin of staphylococci. Susceptible cells, especially platelets and monocytes in humans, have a specific receptor for α-toxin, allowing the toxin to bind, thereby causing small pores through which cations can pass. After binding the toxin, a complex series of secondary reactions occurs, with release of cytokines triggering production of inflammatory mediators. These events cause the symptoms of septic shock that occur during severe *S. aureus* infections (Nilsson et al. 1999).

Beta-toxin is a sphingomyelinase, which damages membranes rich in this lipid. The classical test for beta-hemolysin is incomplete lysis of bovine or ovine erythrocytes (Quinn et al. 1999). This hemolysin allows easy recognition of most, but not all, strains of *S. aureus*, depending on the host species they are associated with and virtually all *S. pseudintermedius* strains from dogs on ox or sheep blood agar. However, the pathogenic role of this toxin is largely unknown.

Gamma-toxin (also called leukotoxin) and leukocidin are proteins that act together to damage

leukocytes and lipid membranes. Few *S. aureus* isolates express leukocidin, but nearly 90% of the strains from severe dermonecrotic lesions and necrotic pneumonia in humans produce this toxin, suggesting that it is an important factor in necrotizing infections (Staali et al. 1998).

Delta-toxin is a very small peptide, produced by most strains of *S. aureus*. Its role in disease is not well known. It has direct and indirect effects on the activity of neutrophils and monocytes, and thus has a pro-inflammatory capacity (Schmitz et al. 1997).

PATHOGENESIS

In *S. aureus*, the expression of extracellular and cell wall proteins necessary for the colonization and invasive phases of infection are regulated through several regulatory loci. These loci modulate in a very complex and efficient way the highly coordinated response of the bacterium toward the changing environments during the infection process. The regulatory agenda is presumably tuned to particular sites in the host organism (Novick 2003).

Mastitis in Ruminants

It is universally accepted that the route of infection in staphylococcal mastitis is via the teat. Staphylococci colonize the tip of the teat, especially when it is damaged or eroded. The organisms pass through the teat duct into the cistern and may subsequently establish in an area of secretory tissue. The pathogenesis of *S. aureus* in the mammary gland most likely involves the generally accepted concept of specific colonization. *In vitro* adhesion of *S. aureus* to ductular and alveolar mammary gland epithelial cells indicates that colonization might be an important step in the development of mastitis (Wanasinghe 1981). *S. aureus* bacteria are furthermore able to bind to extracellular matrix molecules. It is suggested that staphylococci might use matrix proteins—exposed by microlesions or appearing in blood clots—as substrates for adhesion as a step in colonization and the development of mastitic infections. Staphylococci isolated from bovine mastitis have the ability to bind to fibronectin, fibrinogen, laminin, and different types of collagen (Nelson et al. 1991; Cifrian et al. 1994). Milk is an adequate medium for multiplication of staphylococci. During the course of staphylococcal multiplication, cytotoxigenic substances are produced, which cause infiltration of the mammary gland by neutrophils. Aggregation of neutrophils results in clots in the milk and interalveolar edema. The presence of staphylococci and neutrophils obstructs the lobules, which start to involute. Accumulation of fibroblasts, macrophages, and lymphocytes results in the expansion of the interalveolar connective tissue. The bacteria remain in the alveoli and ducts from where they are intermittently excreted. Local intense multiplication of *S. aureus* bacteria may result in abscesses or granulomata.

Leg Problems in Poultry

Bacterial chondronecrosis (also called femoral head necrosis) in poultry is a disease in which mainly *S. aureus* bacteria are involved. After systemic entrance, the staphylococci may deposit in vascular channels in and around the growth plate of the bones, where they bind to proteins and collagen. Partial occlusion of these channels by bacterial colonies and infiltration of heterophils lead to failure of the local blood supply, which results in microabscess formation and bone necrosis. Mostly, the cartilaginous matrix prevents further spread into the epiphysis (Butterworth 1999; McNamee and Smyth 2000).

Abscesses and Septicemia in Rabbits

In rabbits, *S. aureus* bacteria are able to infect small dermal lesions. Other possible routes of entry may be the umbilical stump in newborn rabbits (Hagen 1963) and the genitourinary tract (Rossi et al. 1995). The mammary gland often becomes infected through suckling young. Contrary to mastitis problems in cattle, where staphylococci enter the mammary gland through the teat duct, in rabbit does, staphylococcosis is mainly a wound infection of the nipples and the tissue surrounding them.

Exudative Epidermitis in Pigs (Greasy Pig Disease)

Exudative epidermitis is caused by virulent isolates of *S. hyicus* that produce exfoliative toxin. Both virulent and avirulent isolates of *S. hyicus* can be present simultaneously on the skin of diseased as well as healthy piglets (Wegener and Skov-Jensen 2006). Skin trauma is considered the most common triggering factor allowing the bacteria to establish in the dermis. Fibronectin-binding proteins on the bacterial surface may be important in bacterial adhesion to fibronectin although the fibronectin-binding capacity of *S. hyicus* is low compared with that of *S. aureus* (Lämmler et al. 1985). The first active line

of pigs' defense, phagocyte-opsonin activity, can be eluded by *S. hyicus* strains through their capsules and protein A. This is followed by the action of exfoliative toxin, resulting in separation of epidermis cells. Rounding and separation of cells of the stratum spinosum in the early stages of infection allow for rapid spread of the bacteria in the epidermis. Reactions produced by the host explain the massive exudation leading to dehydration and eventually to death.

Pyoderma in Dogs

Staphyloccocus pseudintermedius should be considered the cause of pyoderma in dogs (Bannoehr et al. 2007). Exposure of extracellular matrix proteins due to underlying conditions favors colonization and adhesion by certain *Staphylococcus* strains, possessing specific adhesive capacity on single matrix proteins (Cree and Noble 1995). Frequent production of leukocidal toxins (synergohymenotropic toxin) and staphylococcal enterotoxin (SECcanine) by strains from skin lesions suggests that these toxins are important for staphylococcal survival and pathogenesis. Generally, studies on characteristics of strains isolated from lesions and from carrier sites failed to demonstrate consistent differences between strains from these two origins. Most probably, unknown host factors are decisive in the pathogenesis of severe staphylococcal disease in dogs. The fact remains that mainly *S. pseudintermedius* is associated with these conditions. Other species, including highly virulent *S. aureus* strains, usually only transiently colonize such lesions.

TYPES OF DISEASE AND PATHOLOGIC CHANGES

In this section, only the most frequently occurring major types of disease are described.

Staphylococcus aureus Infections in Cattle

Staphylococcus aureus in cattle is mainly involved in intramammary infections of lactating cows. *S. aureus* accounts for 25–30% of these infections. Although they are often subclinical, the infections cause considerable economic losses, particularly milk losses, varying from 10% to 25% of the total yield according to the intensity of inflammation and the stage of lactation. Treatment failures (not due to acquired antimicrobial resistance) are particularly high in multiparous cows with more than one infected quarter. Recurrences are frequent, and culling is commonly the only solution. *S. aureus* mastitis in cattle may be clinical or subclinical, and in its clinical form, the disease may vary from a severe peracute form to a very mild form without general signs of infection. The peracute or gangrenous form results in a severe general illness. Unless treatment of acute infections is successful, the reaction becomes chronic. This chronic reaction may also occur subclinically.

Staphylococcus aureus Infections in Poultry

Staphylococcal disease is common in domestic poultry, resulting in dermatitis, bacteremia, osteomyelitis, arthritis, and synovitis. Lameness due to staphylococcal disease has a high economic impact on the poultry industry. The incidence of the disease is variable and depends on environmental factors and management practices (Zhu and Hester 2000).

The lesion traditionally associated with staphylococci in poultry is "bumblefoot." This localized lesion of the foot arises from the penetration of a foreign body followed by secondary invasion by *S. aureus*, but often many different bacteria are involved, so that this condition cannot be considered a true staphylococcal infection.

Localized staphylococcal infection may also occur in association with "battery blister," an abrasive condition of the muscles covering the sternum, or with skin lesions on the wing tips or pelvic region. Litter condition and moisture content appear to be critical to the incidence of localized skin lesions (Butterworth 1999). Recently hatched and hatching chicks with open navels and immature immune systems can be easily infected, leading to mortality and chronic infections very early after hatching (Skeeles 1991). If *S. aureus* gains entrance to the circulation of older birds, a septicemia develops. If the birds do not die in the early septicemic phase, the circulating staphylococci may induce inflammation in many sites throughout the body. Lesions consist of arthritis, tenosynovitis, osteomyelitis, bacterial chondronecrosis, endocarditis, and localized organ abscesses or granulomata (Butterworth 1999). *S. aureus* is one of the major causes of leg weakness in broiler flocks.

Staphylococcus aureus Infections in Rabbits

At the rabbit flock level, two types of *S. aureus* infection can be distinguished: the first type is caused by low virulence strains, affecting only a limited number of rabbits in the flock, and the

second type is due to high virulence strains, causing epidemic spread of disease in rabbitries. The latter type gives rise to chronic problems of staphylococcosis in commercial rabbitries.

Clinical signs and lesions differ depending on the age of the affected rabbits. Many different problems associated with outbreaks of staphylococcosis have been described (Okerman et al. 1984; Matthes 1995; Rossi et al. 1995). Newborn hairless rabbits may suffer from exudative dermatitis with superficial pustules. Generally, the whole litter is affected and high mortality is seen. In older young, subcutaneous abscesses, conjunctivitis, and purulent rhinitis are noticed. Subcutaneous abscesses and pododermatitis frequently occur in broiler rabbits and in does. In rabbits of all age categories, internal abscesses (e.g., in lungs and liver) may be demonstrated. Metritis in does, arthritis, parodontitis, sinusitis, and otitis media may also be seen in association with septicemia. Mastitis in does may vary between an acute gangrenous form ("blue breast") and a chronic form with abscess formation.

Exudative Epidermitis in Pigs (Greasy Pig Disease)

Although *S. hyicus* infections in pigs commonly only affect the skin, the condition may cause serious losses. Initially, the pigs have yellowish-brown crusts on the face and the ears. When the disease becomes more severe, the skin feels greasy and becomes covered with a dark-brown coating in which dirt easily is entrapped. This coating is often localized, in the form of crusts. At this stage, the lesions may heal rapidly, or the exudates may increase, with the crusts becoming confluent, blackened, hard, and firmly adherent, with cracks or furrows. Erosions may be seen on the snout and foot pads. When this stage is reached, death usually follows. It is associated with severe dehydration.

Pyoderma in Dogs

Staphylococcus pseudintermedius rarely causes systemic disease in dogs or other animals. Pyoderma is a frequently occurring condition starting with papular eruptions progressing into pustule formation: small intraepidermal abscesses. Papules remain as small, reddened lesions in cases of bacterial folliculitis. In other cases, furuncles may develop. These deep skin lesions start as folliculitis. Intact furuncles are large hemorrhagic bullae. When the lesions rupture, fistulous tracts with pus formation become evident.

INTERACTIONS BETWEEN THE BACTERIUM AND HOST DEFENSES

The skin and mucous membranes are excellent barriers for preventing invasion by bacteria, such as staphylococci. However, after the skin or mucous membranes have been damaged, the main host defense against a staphylococcal infection consists of the attack and phagocytosis of the bacteria by polymorphonuclear leukocytes (PMNs). This is the case in humans but has also been extensively studied in bovine mammary gland infections. In the initial steps, *S. aureus* gains entrance to the mammary gland tissue through adhesion to epithelial cells (Frost 1975), underlying alveolar myoepithelial cells (Lammers et al. 1999), and extracellular matrix proteins, such as collagen and fibronectin (Kuusela 1978; Mamo et al. 1988). Generally, after recognition of bacteria by PMNs, ingestion and intracellular destruction of these bacteria take place. For *S. aureus*, however, PMNs are not always able to eliminate the infection. This may be due to certain antiphagocytic factors produced by *S. aureus*. These factors include protein A, which has an adverse effect on opsonization, and the formation of a capsule, which masks bacterial surface antigens. Furthermore, it has been shown *in vitro* that *S. aureus* may escape from host defense mechanisms by invading host mammary epithelial cells and myoepithelial cells and even by replicating inside these epithelial cells (Almeida et al. 1996; Lammers et al. 1999).

Cytokines and other proinflammatory factors may aid in the host defense against staphylococcal infection (Soell et al. 1995), but their role has not exactly been defined. In contrast, a massive release of cytokines leading to severe disease may be induced by expression of staphylococcal SAgs, such as enterotoxins and the TSS toxin, which elicit activation of over 25% of all T cells in the body (Fraser et al. 2000).

Influence of Predisposing Factors

Preventive control of *S. aureus* and other staphylococcal intramammary infections in cows is based on two types of measures. Hygiene during milking is important to limit the spread of intramammary infections in herds. Furthermore,

systematic antibiotic treatment by the intramammary route is given at drying-off to cure chronic subclinical intramammary infections established during lactation and to prevent new infections during the dry period.

In poultry, predisposing factors for *S. aureus* disease comprise sharp objects in areas where poultry are reared as well as those with poor litter quality, which enhances footpad ulceration. Particular attention should be given to hatchery management and sanitation since incubators and hatchers are ideal for bacterial growth. The study by McCullagh et al. (1998) reveals an association between disease in broiler chickens and a predominant strain type. Furthermore, these same strains were present in the hatchery, which indicates that the hatchery is a potential source of infection for clinical broiler disease. There may be points in the production cycle where improved hygiene practices could reduce or eliminate the initial incidence of *S. aureus* on day-old chicks, thereby decreasing the risk of clinical disease in broiler flocks. Immunocompromising effects, such as viral infections with infectious bursal disease and chicken anemia virus, may promote the development of systemic staphylococcal infections (Butterworth 1999; McNamee et al. 1999). Vaccination against infectious bursal disease virus and chicken anemia virus is recommended for reducing the risk due to immunosuppressive viral infections (Skeeles 1991).

In rabbits, *S. aureus* bacteria invade traumatic lesions that may be due to poor quality cage wire floors or to fighting between animals. Hereditary factors and the animals' body weight also play a role in developing pododermatitis due to high or low virulence *S. aureus* strains. However, rabbit breeds used today in commercial rabbitries are fairly resistant to these lesions and pododermatitis due to high virulence *S. aureus* strains are also found in relatively young animals, which may suggest that these strains have a higher ability to cause pododermatitis (Okerman et al. 1984).

In piglets, ectoparasites and traumatic lesions from fighting, unclipped teeth, rough bedding, or pen walls may allow *S. hyicus* to establish infection (Wegener and Skov-Jensen 2006).

In dogs, the development of dermatological problems associated with *S. pseudintermedius* is related to anatomic causes, such as skin folds, physical predispositions (e.g., dogs exposed to traumatic injury), and clinical features (e.g., short-haired dogs predisposed to folliculitis; Bensignor 2001). Immunological predispositions or defects are probably important, but the underlying conditions are still largely unknown.

IMMUNITY AND ITS IMPACT ON PATHOGENESIS

It has been stated that finding a vaccine for staphylococci will be quite difficult since protective immunity to staphylococcal infections does not appear to exist at a significant degree. This may be partly due to the fact that in most animal species, the immune system is in constant contact with staphylococcal antigens as many strains are commensal organisms (Otto 2008).

For *S. aureus* intramammary infections in cattle, numerous vaccination attempts have been made with live or killed bacterial cells, isolated bacterial cell walls, toxoid, or killed cell–toxoid preparations. In several experiments, a reduction in the frequency and severity of clinical intramammary infections has been observed. Promising results with vaccines based on fibronectin-binding and fibrinogen-binding proteins have been described in mice (Mamo et al. 1994a, 1994b). This is also true for vaccines containing a combination of alpha-toxoid and recombinant collagen-binding protein, although vaccination with collagen-binding protein or alpha-toxoid alone did not induce protection against challenge in a mouse mastitis model (Mamo et al. 1994a, 1994b, 2000). Tests with these vaccines in cows have not been described. Several commercial vaccines have been marketed, but effective protection of these vaccines against *S. aureus* disease in the field has not been proven (Leitner et al. 2003; Middleton et al. 2006). Recently, in a small trial, promising results were described with a recombinant SEC-producing *S. aureus* strain for the protection against subclinical *S. aureus* mastitis in cows (Chang et al. 2008). However, whatever the antigenic preparations used, complete protection against infections in cows has never been achieved.

Vaccination has also been suggested as a possible method to control rabbit staphylococcosis. Immunization with purified staphylococcal alpha-toxin has been reported to reduce blue breast or gangrenous mastitis caused by *S. aureus* to a less severe form of mastitis (Adlam et al. 1977). However, this immunization was not able to prevent the abscess-type or suppurative mastitis, the form that is seen

in the majority of cases of rabbit staphylococcal mastitis. Studies have been described in rabbits with bacterins against staphylococcosis. These vaccines may have a certain effect on rabbits' immunity against *S. aureus* under experimental circumstances, but in practice their usefulness has not been proven.

Vaccination of sows during pregnancy with autogenous vaccines prepared from both bacterial cells and culture supernatant containing exfoliative toxins of *S. hyicus* may be of value in protecting their piglets against exudative epidermitis (Wegener and Skov-Jensen 2006).

Autogenous staphylococcal bacterins have been administered using various protocols for treatment of dogs with pyoderma. However, controlled studies for evaluation of efficacy are lacking, and clinical impressions of effectiveness have ranged from 0% to 80%.

CONCLUSIONS: NEW DEVELOPMENTS

The immunological response to staphylococci is complicated. Several attempts have been made for the development of staphylococcal vaccines, based on different antigens. However, no vaccine has elicited complete protection against staphylococcal infections. A study making use of whole genome sequence information of *S. aureus* and of individual human sera identified 60 proteins as antigenic in *S. aureus*. Most of these proteins are located or predicted to be located on the surface of the bacterium, or secreted (Etz et al. 2002). Identification of the roles of these antigens in pathogenesis and of their presence in staphylococci infecting animals may facilitate formulation of new vaccines, based on specific antigens that are not only important in pathogenesis but also highly antigenic. Another possible route for the prevention and treatment of staphylococcal infections includes the development of peptides inhibiting staphylococcal virulence gene regulator systems, such as *agr* (Dufour 2000). It has also been suggested that the use of genomics and proteomics could lead to the identification of bacterial surface proteins that are expressed *in vivo* and that might elicit protective immune responses (Zagursky and Anderson 2008). This approach has, for instance, led to the identification of IsdB, a novel vaccine candidate protein for vaccination of humans against *S. aureus*, which has currently been tested in a phase I trial (Kuklin et al. 2006). Considerable further research is required, however, before effective preventive measures against staphylococcal infections can be achieved.

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6
Bacillus anthracis

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INTRODUCTION

Anthrax is the disease caused by the spore-forming bacterium *Bacillus anthracis*. It is principally a disease of herbivorous animals, although, under appropriate circumstances, probably any mammalian species and at least some avian species can be infected. As reviewed elsewhere (WHO 2008), prior to Sterne's development of an effective veterinary vaccine in the 1930s and the later availability of antibiotics, anthrax was one of the foremost causes of uncontrolled mortality in livestock and wild animals globally. The disease remains enzootic in the warmer climes of most countries of Africa and Asia, a number of countries of southern Europe and several parts of the Americas, also occurring sporadically in many other countries where it has been introduced through trade in spore-laden animal products from enzootic areas (see the Handistatus II database in the Reports of the Office International des Epizooties [OIE]).

The cycle of infection of anthrax involves uptake of the spores by the animal host from the environment, their germination within the infected host and multiplication of the vegetative form to large numbers terminating in a massive bacteremia and toxemia. At death, the shedding of the bacilli back into the environment and subsequent sporulation completes the cycle. The events within the host, including the host response, that are essential to the cycle and therefore to the maintenance of the disease globally, are the particular focus of this chapter. The influence of external factors on these events is also covered.

ETIOLOGY, ECOLOGY, AND EPIDEMIOLOGY

Pertinent Literature

Relevant citations supporting statements in this section are again given in WHO (2008).

Etiology, Ecology, and the Cycle of Infection

Anthrax is a bacterial disease primarily affecting herbivores; carnivores and omnivores normally contract anthrax from eating meat from herbivorous victims of the disease. Within the infected host, the organism takes the vegetative form exclusively. At death the vegetative bacilli are shed into the environment either by hemorrhage through the orifices or when the carcass is opened by scavengers, or both, and under the combined conditions of exposure to the oxygen of the air, absence of nutrient, and other adverse conditions, these commence sporulation. The spores are first detectable at about 4–10 h, and sporulation is complete at about 24–48 h, depending on prevailing conditions. The vegetative cells are not hardy and must sporulate or die; in practice, the result is those that successfully sporulate and survive are a small proportion of those shed, the precise proportion again dependent on the environmental conditions (e.g., temperature, water activity, sunlight, organic content). The terminal hemorrhage is presumably the result of the toxin-induced disruption of the vasculature, which is discussed in a later section.

The spores, which can persist in most natural environments for many years, await uptake by another grazing host, which may occur any time from the same day to several decades later. Within

the newly infected host, germination and multiplication commence, ultimately killing the host and completing the cycle. Flies appear to play an important role in large outbreaks in enzootic areas; as well as the more obvious transmission by biting flies, nonbiting flies in some regions can transmit infected blood from an animal that has died of anthrax to leaves of nearby shrubbery, which then become the source of infection for browsing herbivores.

It follows from this generalized cycle of infection that the degree of environmental contamination, and hence the likelihood of one case leading to another, is dependent on factors affecting the rate and extent of sporulation of the shed bacilli. These, in turn, are dictated in a complex manner by temperature, humidity, water activity, pH, oxygen availability, sunlight, and the presence of certain cations, particularly Mn^{2+}. Persistence of the organism in the spore state is also influenced by water activity, temperature, pH, and the presence of nutrients and germinants. High pH due to high calcium content in the soil has long been associated with "favorable sites" for the persistence of anthrax spores. Calcium itself may be the active factor, possibly minimizing loss of Ca^{2+} from the spore core, where it is integral to maintaining the vegetative cell genome precursors in the dehydrated state.

The extent to which *B. anthracis* ever multiplies in the environment is a debated issue. In the presence of artificially supplied nutrients, multiplication and resporulation can happen; however, it is clear that the vegetative forms are not hardy, and, if germination occurs without adequate nutrients or protective factors, such as a high protein content, the vegetative cells would be expected to die rapidly. Most natural environments lack the levels of nutrient or protective factors necessary to induce multiplication. Recently, interest has arisen in the idea that multiplication in the rhizosphere of grasses may be possible, but this has yet to be demonstrated in field conditions.

Another theory is that the frequent association of outbreaks with rain ending a period of drought with animals grazing new vegetation is best explained in terms of a concomitant burst of growth of *B. anthracis* in the soil. Furthermore, the fact that contamination levels at a carcass site can remain seemingly undiminished for years after the death of the animal despite exposure of the site to adverse elements is hard to explain in terms other than periodic localized multiplication. The ecological/epidemiological patterns of anthrax in the Kruger National Park, South Africa, and northern Canada bison were considered to adhere broadly to the concept of "incubator areas"—depressions in calcareous or alkaline locations that collect water and decaying vegetation, which, in turn, theoretically provide a medium permitting germination of anthrax spores and multiplication of the emergent bacilli.

Seasonality; Enzootic versus Sporadic Status

As indicated above, anthrax is a seasonal disease, with onset typically associated with the end of a period of drought, although patterns vary in detail according to the conditions in different locations.

Primary among these conditions is climate, which acts by (1) influencing the way in which an animal makes contact with the spores, through, for example, grazing closer to the soil in dry periods when the grass is sparse, (2) affecting the health of the animal and its level of resistance to infection, and (3) affecting sporulation and germination as already alluded to. Point (2) is relevant to the pathogenesis of the disease but needs to be viewed in the wider context of the other points for a full understanding of the disease.

Enzootic areas generally occur in warmer climes, but notable exceptions are the bison sanctuaries of the Northwest Territories in Canada, and the caribou country in the Taymyr Peninsula, Northern Siberia. The general association with warmer climes is probably attributable to the relationship between temperature and water activity and rates of sporulation of anthrax bacilli shed from animals dying of the disease. The vegetative form has a poor ability to survive outside the animal host, and its ability to sporulate is temperature dependent. Sporulation may be incomplete or may not occur at all below certain temperatures; in temperate or cool climates, where the disease generally arrives through trade and importation of infected animal products, it can be expected to die out with time if further importation is prevented.

The relationship between climate and germination is probably also important. As already discussed, above certain temperatures, with other conditions being appropriate, germination may be induced, but, in the absence of adequate nutrient or other poorly defined factors, the emerging vegetative cells will die out. In the case of colder enzootic regions, some workers hypothesize that persisting anthrax may be the result of spore concentration through being washed by rain into wallows visited

by bulls each year. The possibility is also raised that subclinical infection may also occur, leading to outbreaks when the animals become stressed under summer conditions of diminished food and water sources, crowding around the remaining sources, heat, high concentrations of insects, and hormonal changes heralding the onset of the rutting season. In addition, *B. anthracis* in northern Canada have been found to have higher *in vitro* growth and sporulation rates, which may represent an adaptation for the colder climate, where carcasses cool faster and time after death of the animal for maximal growth and sporulation of *B. anthracis* is limited.

In those regions where flies appear to play a significant role in anthrax transmission, the seasonality of anthrax follows that of the flies. In non-enzootic areas of the world, the seasonal occurrence may reflect patterns of importation of animal products rather than the local climate, although once the disease is established in the importing country, outbreaks are again most likely in the warmer months.

Epidemiology and Transmission

Host Range, Susceptibility, and Infectious Dose

A clear concept of "infectious dose" is crucial to understanding what chance an animal has of developing anthrax. *B. anthracis* is non-invasive and the organism (generally in spore form) needs to reach the internal tissues to establish infection and the chance of infection in an animal is largely determined by the chance of the organism being able to do this. Susceptibility is largely judged in terms of parenterally administered infection, and the result is a spectrum of susceptibility from the more susceptible herbivorous animals to the quite resistant carnivores. Experimental tests in susceptible (herbivorous) animals show that, while LD_{50}s by subcutaneous, intramuscular, or intravenous injection are in the order of <10 to a few hundreds of spores, LD_{50}s by oral or inhalation routes are tens of thousands or many millions. Relative resistance among omnivorous or carnivorous species is apparent with parenterally administered infectious doses, but there is a much less marked difference between susceptible and resistant species when it comes to ingested or inhaled doses needed to produce lethal infection.

Just how animals acquire anthrax is still largely surmise. It is generally difficult to relate experimental dose data with exposure levels that are likely to be encountered by animals in the natural situation. The traditional view is that, when grazers ingest the spores in the soil, grit or sharp plant material that are taken in simultaneously cause sufficient abrasions within the alimentary tract for the spores to lodge and initiate infection. In the case of browsers, infection is believed to be contracted by eating plants contaminated by flies that have fed on anthrax carcasses. It seems feasible that spores may be inhaled when animals graze over a contaminated site during dusty conditions, and biting flies are thought to be responsible for cases and outbreaks in some instances. However, the likelihood of uptake of grit and spiky grass, or of flies carrying infectious doses does not always correlate with case and outbreak circumstances.

Transmission

Direct animal to animal transmission does not occur except in the case of scavengers feeding on anthrax carcasses or, more rarely, animals licking bones for their mineral needs (osteophagia). Sporadic cases are generally point-source infections. The means of natural transmission in outbreaks (as opposed to transmission due to human activities) are frequently not known but may often be fly-borne.

Transmission as a result of trade in animal feeds and products (hides, wool, bones) was a serious transnational issue up until around the 1960s when better control programs in both the enzootic exporting countries and the importing countries reduced the chances of the products being contaminated with anthrax spores. Animal feeds containing contaminated ingredients caused cases directly. In the case of animal products, particularly hides for the leather industry, contaminated effluent from the processing plant arriving on the land downstream gave rise to livestock cases. Very frequently incidents and outbreaks associated with this type of source follow disturbance of soil or watercourses in the vicinity by digging or dredging streams or ditches.

Apart from this, and the beliefs of workers in the Kruger National Park and northwest Canada that spores are washed by rain from carcass sites to concentrate in depressions that become sites of infection, the dilution effect normally to be expected in water renders it unlikely to carry infectious doses and to be a transmitter of the disease. Also, vegetative forms shed by the freshly dead animal falling into water would not be expected to survive well

and would therefore not likely result in the water carrying a substantial dose of spores. Likewise, although the infamous accidental release of anthrax spores in 1979 from a research facility in Sverdlovsk, former USSR, resulted in numerous livestock deaths down wind, wind is unlikely to be able to carry infectious doses in the natural situation.

A long-recognized characteristic of anthrax is that outbreaks in mixed populations usually affect only one species in the affected area while the incidence in other, equally susceptible species in the vicinity remains sporadic. Sometimes there may be a fairly simple explanation for this. For example, in wildlife areas of southeast Africa, the kudu is the most commonly affected species in outbreaks. These regions are frequented by blowflies that feed on the blood and fluids of dead animals and then fly to nearby vegetation contaminating the leaves with droppings or by regurgitation. Kudu are browsers. In southwest Africa, which is unfrequented by blowflies, kudu are infrequently affected and grazers, such as zebra and springbok, are the species most affected. Frequently, however, the reasons why one species has been affected massively and others only marginally remain unclear and are apparently associated with undefined ecological subtleties. As yet, there is no evidence to suggest that strain differences lie behind, or contribute to, this phenomenon.

Deliberate Release—Bioaggression. Anthrax tends to top the list of potential bioaggressive agents due to the hardiness of the spores and their ability to survive adverse conditions during delivery to the target, and their ability to infect via the respiratory route and cause systemic disease with high mortality when used as a weapon. There is little doubt that targeting of livestock could have devastating consequences, and it is by no means a new idea. During World War I, anthrax and glanders were administered to animals in neutral countries (Argentina, Norway, Romania, Spain, and the United States) under German auspices, using needles dipped into cultures, to disrupt the shipment, principally of horses destined for military use, to Germany's enemies. No data exist on the level of damage inflicted, although it was apparently considered a "success" by the German army. During World War II, allied belief that Germany was developing anti-livestock biological warfare (BW) capability led to a British retaliation-in-kind program. Between September 1942 and April 1943, 5 million cattle cakes, each embedded with a capsule containing 5×10^8 anthrax spores, established as the minimum infectious dose (MID) for cattle, were prepared in readiness to target Germany's wartime agriculture section. The cakes were never used and were destroyed shortly after the war.

Suspicions are occasionally aired in the public press that the large epidemic in (then) Southern Rhodesia (now Zimbabwe) at the end of the 1970s, affecting untold numbers of cattle and other livestock and thousands of humans, was due to deliberate release. However, the official stance has always remained that this was a natural outbreak resulting from veterinary services breakdown under the existing insurgency conditions at the time.

SYMPTOMATOLOGY AND DIAGNOSIS

Typically, the first sign of anthrax in animals is sudden, unexpected death, and among the differential diagnoses that may need to be considered are lightning strike, snakebite, chemical poisoning, or ingestion of toxic plants. Minor symptoms, such as mild anorexia or reduced milk production in the case of cattle, may be recalled retrospectively, but these are not invariable. In the last few hours, a steep rise in temperature is typical in at least cattle. Bleeding from the orifices at death again is frequent in many species but not invariable; when seen, however, it should lead to a strong suspicion of anthrax. Edematous swellings of the face and neck may be apparent, especially in more resistant species.

Normally the period between onset of visible symptoms and death may be just a few hours in the more susceptible species but may be longer in more resistant species or in vaccinated animals when the protection afforded by the vaccine is waning. The knowledge that the region has a history of anthrax at some time in the recent or distant past may lead to more rapid recognition of new cases that may occur. Anthrax should be considered in the event of sudden deaths in zoos or other captive animal facilities where meat from knackeries is regularly fed to animals. Cutaneous lesions as seen in human cutaneous anthrax are rarely reported in animals.

The incubation period in susceptible laboratory animals is generally 36–72 h. In livestock, it ranges from 1 to 14 days, usually 3 to 7 days, although, for international trade purposes, the OIE incubation period for anthrax is 20 days.

Diagnosis in animals is still most commonly performed by the century-old M'Fadyean smear, which is the microscopic examination of a polychrome-methylene-blue-stained smear of blood or tissue fluid from the dead animal for the presence of the encapsulated bacilli. Immunochromatographic devices for on-site detection of an anthrax toxin component in blood or tissue fluids are an effective alternative, but these have never become widely available. Culture, where possible, is ideal, especially if follow-up studies are needed. Culture is also the best method for old, decomposed carcasses in which the encapsulated bacilli can no longer be found. Premortem diagnosis is generally not carried out and depends on detecting animals in a herd showing temperature rises and dullness once the diagnosis has been made in an index case. Serology is of little value in diagnosis of animal anthrax and is useful mostly as an epidemiological research tool. Polymerase chain reaction (PCR) for the genes encoding the toxin components or the capsule is highly specific, and direct PCR on animal specimens (as opposed to PCR on isolates from such specimens) is increasingly being accepted for diagnostic purposes.

BACTERIOLOGY

The etiological agent of anthrax, *B. anthracis*, is a facultatively anaerobic gram-positive, spore-forming rod, approximately $4 \times 1\,\mu m$, mostly seen in chains of cells of two to several cells in length from the blood of animals, or in long chains in smears from agar or broth cultures. In the presence of oxygen and toward the end of the exponential phase of growth, one ellipsoidal spore, approximately $2 \times 1\,\mu m$ and not swelling the bacillus, is formed centrally or sub-terminally in each cell. Under a high partial pressure of CO_2 and in the presence of HCO_3^-, the vegetative cell secretes its polypeptide capsule, one of the two established virulence factors of *B. anthracis*, and the basis of the M'Fadyean diagnostic smear.

Suspect isolates are generally easy to confirm as *B. anthracis*. Its colonies on blood agar are readily recognized (matt appearance, characteristically tacky, white or grey-white, occasionally with curly tails at the edges and nonhemolytic). The bacilli are nonmotile and susceptible to penicillin and the diagnostic "gamma" phage. They will produce the capsule when incubated in blood or on bicarbonate–serum agar under CO_2. Full virulence of an isolate is generally confirmed by PCR for the presence of the genes for toxin and capsule production. Biochemical tests are not able to contribute usefully to these tests for confirming an isolate as *B. anthracis*.

Under optimal conditions, sporulation, which begins toward the end of the exponential phase of growth, is complete in terms of resistance to UV, heat, and chemicals after 7–8 h. Germination occurs as the result of specific germinants, or nutrients such as amino acids, acting on a trigger site or receptor within the spore. Common germinants include L-alanine and ribosides, or occasionally germination is induced by combinations of chemicals that are not germinants on their own. Several germination operons encoding these receptors have been detected which respond to the presence of various germinants in the environment external to the spore, encoded by genetic loci on the bacterial chromosome, and in a pathogenicity island on the pX01 plasmid (Fisher and Hanna 2005). These may work synergistically, as the triggering of germination by a single germinant alone may require concentrations higher than those found *in vivo*, and there are several pathways dependent on the presence of multiple co-germinants. Five germination pathways have been recognized in *B. anthracis.* Heating the spores to 60–70°C for 10 min or more predisposes them to germination.

Germination is a fairly rapid process with loss of heat resistance in about 10–15 min, dependent on the ambient temperature. Fernelius (1960) found that, following triggering of germination of *B. anthracis* spores by alanine, tyrosine, and adenosine, and as measured by loss of heat resistance, >99% germination had occurred in 8 min at 30°C and in 16 min at 15°C. Studying germination of *B. anthracis* spores in sera from 11 species, Titball and Manchee (1987) found that serum alanine and tyrosine ranged from 0.01 to 0.35 mmol/l and 0.02 to 0.08 mmol/l, respectively, and germination rates varied from 0.26×10^4 to 1.15×10^4 spores/min/ml in the mouse, dog, sheep, rat, pig, and goat, with commencement only after 15 min in the rabbit and horse and failure to germinate in guinea pig sera. Titball and Manchee concluded that there was no evidence of a relationship between germination ability and the innate resistance of an animal to anthrax.

PATHOGENESIS

Events Following Infection—Pathology

As indicated earlier, it is generally believed that *B. anthracis* is not invasive and that, apart from when it is inhaled into the lungs, a lesion is needed by which it can enter the body. However, recent investigation in laboratory animals into the entry of spores and dissemination of bacilli from the initial site of infection suggest that preexisting lesions are not an absolute requirement for *B. anthracis* to enter through the integument and infect the host. Hahn and others (2005) demonstrated that, following high-dose epicutaneous application of spores onto the unshaved, intact skin of mice, *B. anthracis* spores penetrated the intact epidermis, resulting in infection and death, with infective foci detected in the hair follicles.

Until very recently, knowledge of the events at cell level following entry into a lesion dated from studies in the two decades following the World War II in parenterally infected laboratory animals (WHO 2008). Virulent spores injected into the skin of susceptible animals (mice, guinea pigs, or rabbits) were found to germinate and give rise, in about 2–4 h, to a small edematous area containing encapsulated bacilli. This area expanded and became defined by a zone of altered capillaries through dilation, congestion, and swelling of the endothelial cells and diapedesis of neutrophils. Macrophages and fibrin deposits appeared and the efferent lymphatics became dilated. As the bacilli continued to multiply, spreading edema resulted in fragmentation of the connective tissue with necrosis and hemorrhage. Phagocytosis was apparently minimal.

The bacilli are carried through the lymph vessels to the local lymph nodes, where multiplication occurs, releasing a continuous stream of organisms into the efferent lymph vessels and thence to the spleen and other lymphoid tissues, where multiplication continues. In the final hours of life, failure by the reticuloendothelial system to contain the infection results in a terminal bacteremia with doubling times ranging from approximately 45 min in mice to 95 min in sheep, and 115 min in rats. In resistant animals (rats, dogs, pigs), the spores germinated and the emergent bacilli multiplied in a similar manner to that seen in the susceptible species for about 4 h. At this point, they lost their capsules and multiplication ceased; the bacteria died, disappearing by about 70 h. By 26 h, the lesions had become infiltrated with neutrophils and macrophages, and the zone had become necrotic by 72 h. At 10 days, resolution commenced with the sub-epithelial appearance of dense fibrous tissue and epithelial regeneration. The natural resistance of these species was attributed to an anthracidal basic polypeptide containing a large amount of lysine of leukocyte origin.

As indicated previously, infection by the aerosol route may not be of great importance in anthrax as normally contracted by animals. However, animal models have been studied extensively in relation to potential bioagression at various times since the World War II (WHO 2008). Following aerosol infection, hemorrhagic and cellular lesions surrounded by dense masses of fibrin were found in the more resistant pigs and dogs that were absent in sheep and rhesus monkeys. This ability in more resistant species to "wall off" the invading organisms into local foci of infection may inhibit the development of systemic infection. Inhaled spores apparently did not germinate in the lungs but were phagocytosed by motile macrophages, which carried them through the undamaged epithelium to the lymphatics. Germination commenced on the way to, or on arrival at, the tracheobronchial lymph nodes, and the vegetative cells, freed from the phagocytes, then proliferated with ultimate spread through the efferent lymph duct into the bloodstream as before. Some of the bacteria reached the peribronchial lymph nodes within 15 min of inhaling the spores but clearance from the lung was not efficient. Henderson et al. (1956) noted that in rhesus monkeys exposed to aerosolized spores 15–20%, 2%, and 0.5–1% remained in the lungs at 42, 50, and 75 days, respectively, after exposure. This was broadly confirmed later by Friedlander et al. (1993). Although pulmonary lesions and necrotizing pneumonitis have been noted in human and nonhuman primate inhalation anthrax (Abramova et al. 1993, Fritz et al. 1995), it is possible that these pathologic findings resulted from infection at preexisting or experimentally induced pulmonary lesions, and it had been noted previously that preexisting pulmonary lesions may predispose to the establishment of primary anthrax pneumonitis (Brachman et al. 1966).

Events following ingestion are less well described. In older studies (WHO 2008), focal to diffuse hemorrhagic necrotic enteritis was observed in the small intestine of cattle and sheep, and the localized lesions tended to develop in Peyer's patches,

indicating possible M cell involvement in the uptake of the anthrax bacillus. The primary lesion in 18 of 19 experimentally infected steers was an edematous and hemorrhagic area in the small intestine, 4–6 in. long and located about 16 ft from the pylorus (Jackson et al. 1957). Pharyngeal anthrax in swine was believed to arise from penetration of the bacilli through the superficial epithelium overlying the tonsils or in the tonsilar fossae. Certainly, there are few reports of preexisting lesions in records of anthrax pathology in animals, raising the question of whether such lesions are absolute prerequisites for the pathogenesis of anthrax acquired by ingestion.

More recently, Glomski and others (2007b) evaluated the initial tissue sites infected and pathways by which the infection disseminated following cutaneous, gastrointestinal, and aerosol inhalation inoculation. Following subcutaneous injection of the ear, spores rapidly germinated at the site of injection and became metabolically active, and vegetative bacilli then spread within 24 h to the superficial lymph node, draining the ear; bacteria were detected in the spleen and lung within 12 h, suggesting infection of these organs via bacteremic dissemination from the initial site of infection. Intragastric deposition of spores using a rigid feeding needle resulted in laryngopharyngeal infection, which subsequently spread systemically within 24 h. Infection was likely through abrasions in the upper gastrointestinal tract caused by the rigid feeding needle, lending support to the concept that infection in animals consuming spore-contaminated foodstuffs may result from entry via lesions or abrasions in the alimentary tract. Direct intragastric deposition with a flexible feeding tube resulted in bacterial growth in the Peyer's patches throughout the small intestine within 24–33 h, which progressed to the jejunal lymph nodes and eventually to the spleen and lungs.

Nasal exposure to aerosolized spores resulted in infection of nasal-associated lymphoid tissues within 22 h, which spread to the mandibular lymph nodes, and intratracheal inoculation resulted in mandibular lymph node infection and laryngeal infection. These observations corroborate the previous observations discussed above and support the premise that phagocytosis plays a minimal role in establishing infection at many sites. In addition, they challenge the frequently held belief that infection is initiated only after phagocytic cells have transported germinating spores to lymph nodes.

Virulence Factors

The genome sequence of *B. anthracis* indicates that the bacterium has evolved to grow in animal hosts (Read et al. 2003). Compared with the soil-dwelling bacterium *Bacillus subtilis*, *B. anthracis* has more genes that support the use of peptides and fewer genes that are involved in sugar catabolism. The 5.2-Mb chromosome also encodes a number of putative virulence factors, such as hemolysins, phospholipases, proteases, and proteins involved in iron acquisition (Ariel et al. 2003, Read et al. 2003). Additional proteins encoded on the chromosome or the pXO1 and pXO2 plasmids have been identified recently which contribute or are essential to the virulence of *B. anthracis*, such as siderophores, anthrolysin O, and metal cation transport proteins.

The genes for the two main virulence factors, anthrax toxin and capsule, are found on the two large virulence plasmids. The 182-kb pXO1 contains 217 genes, including those that encode the three genes for anthrax toxin: *pagA*, *cya* (edema factor), and *lef* (lethal factor) are located in a 44.8-kb pathogenicity island (Okinaka et al. 1999). Also found on pXO1 is a germination operon, *gerX*, and genes for the transcriptional regulators AtxA and PagR, which regulate virulence. The 95-kb pXO2 plasmid contains 113 genes, including the capsule biosynthetic operon and genes that encode transcriptional activators of this operon, AcpA and AcpB (Drysdale et al. 2004).

Anthrax Toxin

Anthrax toxin is a key virulence factor that is optimally produced under the host-like conditions of elevated CO_2 (≥5%) and temperature (37°C; Fouet and Mock 2006). These conditions increase toxin gene expression through the master virulence regulator AtxA. Temperature affects the synthesis of AtxA such that cells grown at 37°C contain approximately fivefold more AtxA than cells grown at 28°C. In contrast, CO_2 does not affect AtxA levels; the mechanism of CO_2-enhanced regulation of the toxin genes through AtxA is unclear. In addition to environmental conditions, anthrax toxin gene expression is influenced by growth phase, with maximal expression occurring during late logarithmic growth. The transition state regulator AbrB reduces toxin gene expression in the exponential phase by repressing the *atxA* promoter. The level of AbrB decreases during the late log phase, which

leads to increased *atxA* expression and, therefore, increased toxin production.

Transcription of *pagA* mRNA is detected after just 15 min of stimulation of germination (Cote et al. 2005), and *lef* transcription occurs within 3 h of spore phagocytosis and immediately subsequent to germination (Guidi-Rontani et al. 1999). Expression of *pagA*, *lef*, and *cya* appears to be, in part, controlled by a chromosomally encoded quorum-sensing signaling molecule, as does log phase vegetative growth (Jones et al. 2005).

Anthrax toxin is a combination of two binary toxins, lethal toxin (LeTx) and edema toxin (EdTx). Each toxin consists of an enzymatic component, lethal factor (LF, 90 kDa) or edema factor (EF, 89 kDa), and a common cell-binding component, protective antigen (PA, 83 kDa), which transports the enzymes to the host cell cytosol.

PA and Host Cell Receptors

Most cell types can be intoxicated by anthrax toxin because they express one or both of the cellular receptors of PA, ANTXR1, and ANTXR2 (Young and Collier 2007). Both anthrax toxin receptors are type I membrane proteins with an extracellular I domain that binds PA. The I domain contains a divalent metal ion bound to amino acids that form a metal-ion-dependent adhesion site (MIDAS). In the PA–receptor complex, the coordination of the MIDAS metal is completed by an aspartic acid side chain from PA. This type of interaction has been observed in a complex between the α2 integrin I domain and its ligand, collagen, and is presumed to occur between ANTXR1/2 and its natural ligands, components of the extracellular matrix. The natural functions of the anthrax toxin receptors are to promote cell adherence and spreading in angiogenic processes.

Receptor-bound PA is a substrate of cellular furin-like proteases, which cleave a single site in PA to separate the protein into a 20-kDa fragment and a 63-kDa fragment. PA_{63} remains associated with the receptor and co-oligomerizes with other molecules of PA_{63} to form ring-shaped heptamers (see fig. 6.1). Heptameric PA_{63} has also been observed in the blood of intoxicated animals, suggesting that cleavage of PA and subsequent oligomerization of PA_{63} does not occur solely on the host cell surface—it is not clear where processing and oligomerization of PA primarily occurs (in the blood or on the cell surface), and this might depend on the species of animal infected (Moayeri et al. 2007). The heptamers bind a maximum of three molecules of EF or LF on the flat, upper surface of the heptameric ring. Heptameric PA_{63} causes the associated receptors to enter a lipid raft and then be internalized by clathrin-dependent endocytosis.

The acidic environment of the endosome allows loops from the heptamer to insert into the endosomal membrane to form a transmembrane β-barrel and the enzymes to partially unfold into a molten globule state. Translocation of EF and LF through the PA_{63} transmembrane pore is driven by proton motive force. The heptamer further facilitates translocation by acting as a chaperone to keep the enzymes in a translocation-competent state. EF and LF refold after entering the cytosol.

Edema Toxin

Edema factor (EF) is calcium- and calmodulin-dependent adenylate cyclase with a catalytic rate 100-fold greater than that of mammalian adenylate cyclases (Banks et al. 2006). Calmodulin is a mammalian calcium-sensing protein that binds EF and reorganizes its active site to allow binding of adenosine triphosphate (ATP; Drum et al. 2002). The conversion of ATP to cyclic adenosine monophosphate (cAMP) by EF is thought to contribute to pathogenesis in numerous ways. Intoxication of phagocytes by EdTx impairs their ability to internalize bacteria and alters their production of cytokines, which would help the anthrax bacilli to avoid the innate immune response. EdTx kills some types of cells, causes tissue necrosis, and can kill mice. Mice that have died from intravenous injection of EdTx show intestinal intralumenal fluid accumulation and extensive organ damage (Firoved et al. 2005). EdTx also induces a decrease in circulating lymphocyte population (Cui et al. 2007), induces polymorphonuclear neutrophil (PMN) chemotaxis and diminishes the neutrophil oxidative response to bacterial pathogens by inhibiting lipopolysaccharide (LPS) priming and superoxide anion release.

Lethal Toxin

Lethal factor (LF) is a zinc-dependent metalloprotease that cleaves the amino termini from mitogen activated protein kinase kinases (MAPKKs) 1–4, 6 and 7 (Banks et al. 2006). Cleavage of these MAPKKs prevents them from phosphorylating the downstream MAPKs ERK1/2, p38, and c-Jun NH2-terminal kinase (JNK), and inhibits activation of

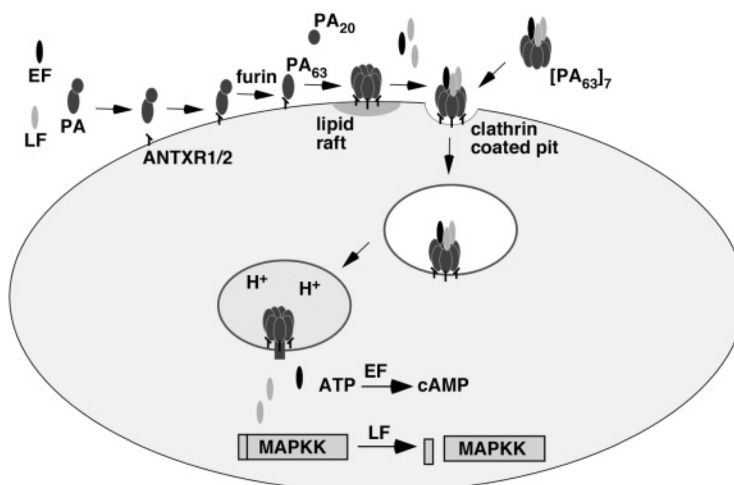

Figure 6.1. Entry of anthrax toxin into mammalian cells. The three components of anthrax toxin are released from the bacterium as soluble monomeric proteins: edema factor (EF), lethal factor (LF), and protective antigen (PA). PA binds the cellular receptors ANTXR1 and ANTXR2 and is then cleaved by furin-like proteases into PA_{20} and PA_{63}. PA_{63} forms heptamers, $[PA_{63}]_7$, that enter lipid rafts and bind up to three molecules of EF and LF. Alternatively, toxin complexes may form before they associate with cells. The complexes are internalized through clathrin-coated pits and are trafficked to acidic compartments (early endosomes and multivesicular bodies) that facilitate membrane insertion of $[PA_{63}]_7$ and translocation of EF and LF through the $[PA_{63}]_7$ pore. EF binds calmodulin and converts ATP to cAMP; LF is a protease that cleaves amino terminal fragments from MAPKKs. A co-receptor, LRP6, may participate in the entry process (not shown). (See color plate)

alternative signaling pathway proteins including activating transcription factor 2 (ATF-2), serine/threonine kinase, and glycogen synthase kinase 3-alpha and beta. It is not known whether LF has additional substrates or whether the cleavage of MAPKKs can account for all of its cellular effects.

One of the first effects of lethal toxin (LeTx) to be described was the rapid induction of death in macrophages from some strains of mice. Recent work has shown that cell death is mediated through Nalp1b (Boyden and Dietrich 2006), a member of a family of NOD-like receptors that includes Nalp3 and IPAF. The activity of LF is somehow detected by Nalp1b, which then likely homo-oligomerizes and binds pro-caspase-1. Pro-caspase-1 is autolytically activated within this complex, termed the inflammasome, and caspase-1 is then competent to cleave other substrates. The only other proteins known to participate in this form of cell death, termed pyroptosis, are the pro-apoptotic mitochondrial proteins Bnip3 and Bnip3L (Ha et al. 2007), but their link to the inflammasome is unknown. Macrophages from other strains of mice are resistant to pyroptosis, yet still are susceptible to being killed through an apoptotic pathway. The ability of LeTx to kill macrophages likely contributes to pathogenesis because mice that have been depleted of macrophages are more susceptible to killing by *B. anthracis* (Cote et al. 2006). Other cell types that are susceptible to killing by LeTx are dendritic cells, neutrophils, and certain kinds of epithelial and endothelial cells, including lung epithelial and microvascular endothelial cells, which likely contribute to pathologic changes in tissue seen in anthrax (Paddle et al. 2006).

In addition to causing death of cells, LeTx impairs a variety of cellular functions that are involved in the immune response. Many of these cellular effects, such as the downregulation of cytokine production, can be attributed to the cleavage of the MAPKKs by LF. MAPKK cleavage also results in a reduction in macrophage production of NO and TNF-alpha

induced by LPS or gamma interferon (IFN). LeTx inhibits the production of Type IIA phospholipase A2 by macrophages, blocks the differentiation of monocytes into macrophages, impairs production of superoxide by neutrophils, and reduces neutrophil motility. LeTx also inhibits the LPS-mediated activation of IFN-regulatory factor 3 (IRF3), which subsequently prevents induction of chemokines including IRF3 which are part of the normal response to viral or bacterial infection (Dang et al. 2004). LeTx additionally inhibits the PMN chemotactic response and inhibits chemokinesis, as a result of impairment of actin filament assembly via disruption of p38 MAP kinase-mediated phosphorylation of Hsp27 (During et al. 2007).

In B-cells, LeTx diminishes the production of immunoglobulin, and LeTx cleavage of MAPKK disrupts Toll-like receptor (TLR) stimulated signaling, blocking the TLR-2 signaling response to gram-positive cell wall peptidoglycan and lipoteichoic acid and TLR-4 response to anthrolysin O. MAPKK cleavage by LeTx also inhibits the B cell proliferative response to IL-4 stimulation, and TLR-2 or TLR-4 stimulated production of anti-IgM and anti-CD40 antibodies, an effect sustained even after LeTx is eliminated (Fang et al. 2006).

Injection of purified LeTx into animals induces shock-like symptoms and death. Death does not appear to be the direct result of macrophage lysis or to be cytokine mediated. Death of mice is associated with hypoxia-mediated necrosis of the liver and with pleural effusions. Pleural effusions were also evident in rats that succumbed to LeTx, but hepatic injury was not. Myocardial dysfunction and disruption of the vasculature are two effects of LeTx that have been suggested to play a primary role in shock (Li et al. 2007). *In vitro*, LeTx induces MAPKK cleavage and cellular apoptosis of the endothelial cells lining the interior of blood vessels, resulting in increased vascular permeability and extravasation of fluid from the circulatory system with increased hemoglobin levels (Cui et al. 2007).

EdTx and LeTx

Toxins EdTx and LeTx disrupt intracellular signaling and stimulation response by both B-cells and T-cells (Comer et al. 2005, Fang et al. 2006). However neither toxin induces lymphocyte apoptosis or cytolysis. Together, LeTx and EdTx inhibit activation and proliferation of CD4+ T cells stimulated with anti-CD28 and anti-CD3 antibodies, and inhibit secretion of cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-17, TNF-alpha, IFN-gamma, and granulocyte-macrophage colony-stimulating factor, also blocking signaling and proliferative responses in B-cells. Unlike LeTx, EdTx does not affect phosphorylation of these signaling kinases, however it inhibits JNK phosphorylation.

LeTx and EdTx both contribute to deterioration of the hemodynamic status of the host and development of cardiovascular shock. In rats, although lethal doses of LeTx are one-tenth of those of EdTx, infusion of lethal doses of EdTx induces hypotension within 2–10 hours, both several hours earlier and to a greater magnitude than does LeTx, and the two toxins exert an additive hypotensive effect. Furthermore, LeTx induces a decrease in heart rate which may contribute to the development of shock (Cui et al. 2007). In addition, LeTx represses glucocorticoid receptor function, which may contribute to the severity of intoxication (Webster et al. 2003).

Capsule

The second major virulence factor of *B. anthracis* is an anionic polypeptide capsule that surrounds the bacillus (Candela and Fouet 2006). It consists of linear chains of poly-γ-D-glutamic acid (PGA) that are covalently attached at one end to the peptidoglycan of the bacterial cell envelope. The polymers appear to be disordered, but may adopt secondary structure depending on the pH of the solution. These long chains, with molecular weights exceeding 400 kDa, are resistant to proteases that cleave at α-amino linkages since the glutamic acid residues are linked through the γ-carbons of the sidechains. PGA is synthesized in the cytoplasm by an ATP-dependent process and is then exported from the cell. The operon responsible for capsule synthesis contains five genes: *capA*, *capB*, *capC*, *capD* (*dep*), and *capE* (Candela and Fouet 2006). CapD is a γ-glutamyltranspeptidase that anchors PGA to the peptidoglycan layer. Cap A, B, C, and E form a membrane-associated complex that is responsible for the synthesis and secretion of PGA. CapB, a member of the tetrahydrofolate synthase family, has an ATP-binding motif and is believed to function as a glutamic acid polymerase. The exact functions of Cap A, C, and E are not known.

Strains of *B. anthracis* that do not produce a capsule are highly attenuated in animal models

because unencapsulated bacilli are more susceptible to being phagocytosed than are encapsulated bacilli. The antiphagocytic property conferred by the capsule is a result of its being poorly recognized by phagocytic cells, perhaps in part because of its anionic charge, and because it shields surface structures that are more readily detectable by the immune system. Immediately underlying the capsule is the surface layer, or S-layer, of the bacillus cell wall, primarily composed of two chromosomally encoded surface proteins, extractable antigen 1 (EA1) and surface array protein (Sap). In unencapsulated *B. anthracis* strains, EA1 induces a strong humoral antibody response; however, capsule inhibits antibody binding to the cell surface. In combination, the capsule and S-layer provide resistance to the binding of complement component C3 and to innate alternative complement pathway-mediated defense (Fouet and Mesnage 2002). The capsule is weakly immunogenic; that is, it may help the bacilli resist the adaptive immune response to an extended infection, such as cutaneous anthrax. It is unlikely, however, that the host would have time to mount an anticapsule antibody response after intestinal or aerosol infection before succumbing to the disease. In addition, *B. anthracis* sheds PGA (Makino et al. 2002), but the impact of soluble PGA on an infection remains to be determined.

Attenuation of unencapsulated strains may result from an inability to spread from the site of infection, as was observed in an inhalation model in which unencapsulated bacteria failed to escape the pulmonary-associated lymph nodes and colonize the spleen. In addition, this inability to colonize the spleen might be due to the large number of immune cells in this organ that would be more efficient at killing unencapsulated bacteria. Anthrax toxin does not affect the lethality of encapsulated strains in certain mouse models, but does affect the host response to the infection and the resulting pathology (Drysdale et al. 2007).

Chromosomally Encoded Virulence Factors

Chromosomally encoded factors play an additional role in virulence and pathogenesis. Fully virulent *B. anthracis* strains such as Ames or Vollum 1B retained virulence in certain mouse models when cured of pX01 and rendered non-toxigenic, whereas less virulent strains had reduced virulence when cured of pX01, even when supplemented with pXO2 plasmids from virulent strains (Welkos 1991).

Unspecified Extracellular Proteases

Using plasmid-cured strains of *B. anthracis*, Stepanov et al. (1999) identified genes near the Met(–) region on the chromosome, which contributed to virulence, including extracellular proteases, which were cytotoxic to macrophage-like cells.

Siderophores

Virulence of *B. anthracis* is dependent on its ability to scavenge iron, which is limiting in the host environment. Iron is acquired by low-molecular-weight chelating agents synthesized by the bacterium, called siderophores, that bind ferric iron and are transported back into the cell. There are two siderophore synthesis gene clusters in *B. anthracis*: *bacCEBF* and *asbABCDEF* (Abergel et al. 2006). The *bacCEBF* gene products synthesize a 2,3-catecholate siderophore (Wilson et al. 2006), bacillibactin, which is not required for virulence in a mouse model but could potentially contribute to pathogenesis in other animal hosts. The *asbABCDEF* pathway synthesizes petrobactin, a 3,4-catecholate siderophore, which is important for growth in macrophages and virulence in mice. The 3,4-dihydroxybenzoyl iron-chelating group of petrobactin is rare in siderophores and allows petrobactin to avoid being bound by siderocalin, an innate immune molecule that sequesters bacillibactin and other bacterial siderophores.

Anthrolysin O

Vegetative *B. anthracis* secrete anthrolysin O (ALO), a cholesterol-dependent cytolysin encoded by the chromosomal BA3355 gene (Shannon et al. 2003, Park et al. 2004). ALO activates TLR4, which is normally considered a signaling receptor specific for gram-negative bacterial LPS, and not for gram-positive bacteria. Through TLR4 activation, ALO stimulates TNF-alpha, IL-1, and IL-6 mRNA production. LeTx-induced macrophage apoptosis is dependent on ALO activation of TLR4, as ALO-deficient strains are incapable of inducing macrophage apoptosis, but in the presence of ALO, LeTx renders macrophages sensitive to TLR4-stimulation-dependent apoptosis by preventing activation of the p38 MAPK pathway.

Manganese Ion ATP-Binding Cassette Transport Protein

The *B. anthracis* genome encodes an ATP-binding cassette transporter lipoprotein (MntA), which is an

ortholog of the *Streptococcus pneumoniae* surface antigen PsaA, and is involved in bacterial uptake of manganese ions. Expression is both *in vitro* and *in vivo*, and is independent of the effect of either the pXO1 or pXO2 virulence plasmid. MntA deletion diminished *in vitro* vegetative growth of *B. anthracis*, increased the susceptibility of bacilli to oxidative stress, and doubled the time required for lysis of macrophages infected with MntA deletion-mutant spores. *In vivo*, deletion of MntA significantly attenuated the virulence of *B. anthracis* despite normal toxin and capsule expression, and resulted in a greater than three-log increase in LD_{50} in the guinea pig (Gat et al. 2005).

Phospholipase C

The *B. anthracis* genome encodes two putative phospholipase C (PLC) proteins at BA0677 and BA3891 with homology to PLCs in *Listeria monocytogenes*, a phosphatidyl-inositol-specific (PI-PLC) and a phosphatidyl–choline preferring phospholipase C (Read et al. 2003). The (PI-PLC) of the *Bacillus cereus group* cleaves glycosylphosphatidylinositol (GPI)-anchored surface proteins inserted into the cell lipid bilayer, such as the CD14 LPS and Fc-gamma-RIII receptors expressed by antigen presenting cells. *In vitro*, PI-PLC demonstrated strong GPI-anchor cleavage activity, reducing the response to TLR2, TLR3, TLR4, and TLR9 ligand stimulation and up-regulation of CD80, CD86, thereby reducing major histocompatibilty complex (MHC) class II surface antigen expression, TNF-alpha production, and MAPK signaling in dendritic cells, which are thereby down-modulated (Zenewicz et al. 2005). *In vivo*, PI-PLC induced suboptimal T cell priming, causing a reduced antigen-specific CD4 T cell response. PLC inactivation attenuates vegetative growth of the bacillus and reduces its cytotoxic effects on macrophages, indicating their functional importance in virulence (Heffernan et al. 2006).

IMMUNOLOGY

Innate Immune Response

Complement

The increased susceptibility noted in complement component 5 (C5) deficient mouse strains and in normally resistant laboratory mice depleted of complement with cobra venom has shown the importance of complement in the nonspecific immune defense against *B. anthracis* infection (Harvill et al. 2005). Complement-containing sera from human cutaneous anthrax patients opsonize vegetative *B. anthracis* and induce oxidative metabolism in neutrophils from normal control donors (Alexeyev et al. 1994). However, the capsule and S-layer resist complement component C3 binding and the alternative complement pathway-mediated defense mechanisms (Fouet and Mesnage 2002), reducing the protective efficacy of this arm of the host immune defense.

The innate immune recognition of *B. anthracis* infection is complex, with involvement of a variety of receptor and signaling pathways.

The extent of the local inflammatory response and leukocytic infiltration has been correlated with the degree of resistance to infection observed in some animal species. Within 2 h of subcutaneous inoculation with *B. anthracis* spores, vegetative bacilli are observed replicating in local tissues, and phagocytes have mobilized to the region of infection and begun to phagocytose spores and bacteria (Welkos et al. 1989).

Polymorphonuclear Leukocytes

In the first hours following either subcutaneous or intraperitoneal inoculation, polymorphonuclear leukocytes (PMNs) comprise the majority of the responding infiltrative phagocytic cell population. By 24 h post infection and thereafter, the population becomes more mixed, with both PMNs and macrophages present. Macrophages respond chemotactically to bacteria-derived attractants and to activated serum with normal C5 complement function, and PMNs are chemotactically attracted by Sterne culture filtrate (Welkos et al. 1989).

Although major constituents of the early localized response to infection in mice, PMNs appear to have a limited role in clearing *B. anthracis* spores and controlling local infection (Cote et al. 2006, Drysdale et al. 2007). Depletion of PMN populations results in only insignificant reduction in survival following intraperitoneal or low-dose aerosol challenge, and PMN augmentation does not improve survival. However, induced neutropenia significantly reduces survival in animals with high-dose aerosol challenge. PMNs phagocytose bacteria opsonized with complement or serum containing antibodies against the bacterial cell wall, inducing oxidative metabolism to destroy the phagocytosed bacteria. Immunization with killed anthrax spores or bacilli enhances this defensive mechanism. However, as

previously discussed, EdTx and LeTx minimize this effect. EdTx inhibits PMN phagocytosis and oxidative metabolism, and LeTx inhibits the PMN chemotactic response and cytokine secretion and increases apoptosis of recruited PMNs.

Macrophages and Dendritic Cells

The involvement of macrophages in spore germination and disease initiation has been studied intensively (Bozue et al. 2007, Cleret et al. 2007). To establish infection and disease, the anthrax bacillus must reach an environment in the host where it is able to multiply efficiently. As discussed previously, subcutaneous inoculation rapidly yields germination and appearance of vegetative bacilli both extracellularly and within phagocytes with subsequent invasion into underlying tissues and intravascular spread in susceptible animals. With inhaled spores, however, the alveolus does not provide a suitable site for germination, and the spore must cross the alveoloepithelial barrier into the body, being carried within macrophages to the pulmonary-associated lymph nodes, where germination and vegetative outgrowth occur, followed by bacteremia and dissemination to the rest of the body.

Where it is involved, phagocytosis of spores occurs via actin recruitment and assembly of F-actin-rich phagocytic cups leading to formation of the phagosome. Disruption of actin microfilament polymerization inhibits the binding and uptake of spores. This reorganization of actin indicates that phagocytosis of spores is mediated by macrophage type I immunoglobulin surface receptors for the Fc portion of IgG, and is enhanced by IgG binding to PA. The spore-containing phagosome fuses with the lysosome to form the phagolysosome, with a tightly adherent interaction between the outermost integument of the spore, or exosporium, and the phagolysosomal membrane (Guidi-Rontani and Mock 2002). The *Bacillus*-collagen-like protein of *B. anthracis* (BclA), a structural component of the exosporium forming hair-like filaments projecting from the surface, may be involved in the interaction of spores with host cells. BclA inhibits nonspecific spore binding to nonprofessional phagocytic cells such as bronchial epithelial cells, directing spores toward uptake by macrophages. BclA-deficient mutants have greater virulence in the mouse model, suggesting that these spores have increased binding to respiratory epithelial cells and reduced affinity to and clearance by macrophages. The carbohydrate rhamnose is also present in the exosporium and appears to enhance macrophage binding and uptake of spores (Bozue et al. 2007).

The spores are stimulated to germinate within the phagolysosome in as little as 10–30 min, and specific nutrients, such as alanine or histidine, trigger germination via the germination operons (Guidi-Rontani et al. 1999). The majority of spores are killed as they germinate within the phagolysosome, but spores remaining dormant are protected by the exosporium (Kang et al. 2005).

Exosporium protection involves several mechanisms, including enzymatic disruption of the generation of reactive oxidative species. Arginase in vegetative *B. anthracis* and in the exosporium compete with macrophage-generated NOS2 for arginine, reducing macrophage production of nitric oxide (NO) and enhancing survivability of the germinated spores and vegetative bacilli (Weaver et al. 2007). A superoxide dismutase on the exosporium surface may also protect germinating bacteria in the phagolysosome (Baillie et al. 2005). Superoxide flux stimulates spore germination and may promote spore germination within the macrophage. The *B. anthracis* genome encodes for a NO synthase (baNOS), which metabolizes L-arginine, N(omega)-hydroxy-l-arginine, and imidazole producing nitric oxide, which may protect germinating *B. anthracis* against reactive oxygen species by suppressing enzymatic reduction of cysteine, inhibiting the re-reduction of ferric iron, which sustains the Fenton reaction, and activating catalase to detoxify excess peroxide, thereby attenuating peroxide-induced DNA damage (Shatalin et al. 2008).

The macrophage, in turn, is susceptible to being killed by newly germinated bacilli since the bacilli express the toxin genes rapidly after germination, releasing anthrax toxin within the phagolysosome (Banks et al. 2005). The previously discussed impaired function of macrophages due to LeTx reduces their bactericidal activity, permitting survival and escape of the germinated bacilli.

Capsule appears immediately after the beginning of germination and may protect bacilli in the phagolysosome. It emerges as blebs from spores during germination, which coalesce to form a continuous layer, encapsulating the vegetative bacilli after they emerge (Fouet and Mesnage 2002).

The outcome of the macrophage–spore interaction depends, therefore, on whether the macrophage is able to kill the bacterium before it is intoxicated.

Only a small fraction of spores may need to germinate and exit from the macrophages to establish infection. The number of spores infecting a single macrophage (multiplicity of infection [MOI]) may be important; the probability for vegetative bacillary growth within the macrophage increases with increased MOI (Kang et al. 2005). It is unclear whether the bacteria possess specific factors that facilitate their escape from the phagolysosome.

Dendritic cells also take up *B. anthracis* spores by coiling phagocytosis and macropinocytosis within 30 min of inhalation exposure by dendrite extension across the alveoloepithelial barrier, with subsequent migration to the thoracic lymph nodes (Brittingham et al. 2005, Cleret et al. 2007). Dendritic cells bridge the innate and adaptive immunity, phagocytosing, processing, and presenting antigenic material on MHC molecules, thereby activating naive lymphocytes in the lymph nodes. The dendritic cells undergo maturation subsequent to spore uptake, as evidenced by the down-regulation of tissue-retaining chemokine receptors and the up-regulation of the chemokine receptor CCR7, which are required for migration to lymph nodes. Within hours LeTx and EdTx impair dendritic cell secretion of TNF-alpha, IL-1alpha, IL-6, IL-8, IL-10, and IL-12. LeTx also inhibits up-regulation of costimulatory molecules CD40, CD80, and CD86, which are essential to induction of adaptive immunity, resulting in impaired ability of the intoxicated dendritic cells to stimulate Th1 and Th2, and to prime CD4+ T cells or naive T cells, and the T cells that may be initially activated fail to mature into memory cells (Agrawal et al. 2003, Brittingham et al. 2005). LeTx will kill dendritic cells by inducing slow apoptosis over the 72 h after intoxication.

Spores are also internalized by fibroblasts and epithelial cells *in vitro* via a process resembling the zipper mechanism used by invasive bacteria to invade nonphagocytic cells (Russell et al. 2007). This suggests that nonphagocytic invasion is mediated by interaction between surface proteins on the bacilli and receptors on the host cell surface. Two chromosomal genes encoded for on the *B. anthracis* genome, BA0552 and BA1346, encode putative surface proteins resembling internalin proteins in *L. monocytogenes*, which mediate entry of *L. monocytogenes* into nonphagocytic host cells and which may be involved in the process of *B. anthracis* invasion of nonphagocytic cells (Read et al. 2003).

Once internalized, vegetative bacilli escape from membrane vacuoles into the cytosol (Russell et al. 2007). Although fewer spores might be internalized by nonphagocytic cells *in vivo*, the less bactericidal intracellular environment of nonphagocytic cells might allow more bacilli to escape.

The site of entry of spores may influence the cell type in which germination occurs, and as discussed earlier, more than one cell type may be involved in establishing an infection. Furthermore, heterogeneity occurs in the manner of spore uptake by macrophages from different sources; for example, spore uptake by alveolar and peritoneal macrophages may involve different classes of phagocytic receptors and uptake mechanisms (Stojkovic et al. 2008). Also, germination can occur at some sites, depending on the route of infection.

The Interferon System

The IFN system, comprised of alpha/beta IFN and gamma IFN, is an important modulator of the innate immune response, and this response is targeted by the anthrax toxins, as described previously. Exposure of spore-infected macrophages to exogenous alpha/beta IFN and gamma IFN significantly improves cell viability and reduces the number of germinated intracellular *B. anthracis* spores. Gamma IFN, secreted by NK cells and T lymphocytes, activates phagocytes, modulates chemotaxis, and up-regulates antigen presentation, promoting a Th lymphocyte type-1 response demonstrating increased killing of *B. anthracis* spores and survival of LeTx intoxication (Gold et al. 2004, Kang et al. 2005). CD4+ T lymphocytes secrete gamma IFN strongly when incubated with macrophages and killed spores, and depletion of gamma IFN from mice protected by CD4+ T cell transfer eliminates the conferred protection. The CD4+ T cell and gamma IFN response was also shown to play a significant role in the protective cellular immune response against encapsulated *B. anthracis* infection (Glomski et al. 2007a). Gamma IFN therefore plays an essential role in both the innate and cell-mediated immune response.

Alpha/beta IFN and gamma IFN stimulate induction of genes for antibacterial cellular products including inducible NO; LeTx disrupts IFN signaling by inhibiting alpha/beta IFN and inducing STAT1 phosphorylation in alveolar macrophages, thereby diminishing macrophage effectiveness (Gold et al. 2004).

Adaptive Immune Response

The roles of dendritic cells and IFNs in protection against anthrax have already been discussed. Passive immunization with serum containing antispore antibody fails to provide protection, indicating that humoral immunity to spore antigens was not sufficient for protection. Depletion of CD4+ T cells in spore-immunized mice reduced their resistance to challenge infection, while transfer of CD4+ T-cells from killed spore-immunized mice to naïve mice conferred protection, indicating that the CD4+ T lymphocytes are required for any protection induced by spores (Glomski et al. 2007a).

It is now well established that PA is the principal protective antigen in protection against anthrax. Immunization with PA enhances B cell antibody production against both LF and EF, compared with immunization with either LF or EF alone. PA is a T cell-dependent immunogen, and immunization with adjuvanted recombinant PA (rPA) induces a strong IgG antibody response composed primarily of subclass IgG1, indicating control by Th2-directed helper CD4+ T cells with both Th1 and Th2-CD4+ cells involved in the recognition of PA epitopes (Zhang et al. 2008).

Serological correlates of protection based on anti-PA and toxin neutralizing antibody titers have been determined in laboratory animal species including rabbits and guinea pigs, for use in evaluating antibody response and protection following immunization with PA or rPA. However, the duration of this immunity may be limited (Pittman et al. 2002), and anti-PA and anti-LF titers may not directly correlate with protection against challenge.

Immunization with live spore veterinary vaccine induces strong humoral and cell-mediated immune reactivity in guinea pigs. Four weeks after a three-dose immunization series, serum IgG levels against vegetative *B. anthracis* antigen extracts were significantly elevated compared with nonimmunized controls. The antigenic extracts additionally induced a strong cell-mediated response, as measured by both skin testing and antigenic stimulation of splenocyte proliferation measured 28 days after immunization in human subjects (Shlyakhov et al. 1997).

Effects of Immunization

The Sterne live spore livestock vaccine, developed in the 1930s, and its counterparts in the Russian sphere of influence proved to be very effective in the control of anthrax throughout the world (WHO 2008).

The high degree of effectiveness of these vaccines may be attributable to protective epitopes present on the spores or outgrowing vegetative cells. Immunization with the combination of formaldehyde-inactivated spores and PA induced both significant anti-PA and antispore titers, and provided complete protection against virulent *B. anthracis* challenge in mice and guinea pigs; inactivated spores alone elicited antispore antibody titers and provided partial protection in mice and complete protection in guinea pigs (Brossier et al. 2002). However, Turnbull et al. (1988, 1990), using a number of agents unrelated to *B. anthracis*, showed that the enhancement of PA protection by the supplements was not dependent on the supplements being, or containing, *B. anthracis* antigens.

Immunization enhances the effectiveness of the innate immune response. Anti-PA IgG binds to the spore surface, and pretreatment of spores with anti-PA antibodies enhances phagocytosis and spore killing by the phagocytes. PA can be detected on the surface of germinating spores, but not on purified ungerminated spores, and transcription of *pagA* mRNA is not detected in ungerminated spores, but can be detected just 15 minutes after germination is triggered. Still, anti-PA antibody will bind to the surface of ungerminated spores, as well as germinated spores presumably due to PA or anti-PA antibody-reactive antigens on the spore surface (Cote et al. 2005).

Anti-spore antibodies also delay spore germination *in vitro* (Cote et al. 2005) but do not impart resistance to the germinated encapsulated bacteria (Stepanov and Leppla 1996). Sera from animals immunized with germinated spores were more inhibitory than sera from animals immunized with ungerminated spores, suggesting the presence of protective antibodies against antigens only on the germinated spores.

Efforts to identify other potential antigens on the exosporium which enhance the immune response have led to the identification of five major immunogenic exosporium proteins, including an alanine racemase, superoxide dismutase, and BclA. BclA is the immunodominant glycoprotein antigen on the exosporium surface and generates a strong reaction with antibodies in polyclonal sera from mice immunized against *B. anthracis*. BclA may augment the immune response; spore opsonization with

anti-BclA antibodies significantly increased uptake by macrophages and inhibited germination within macrophages, and although BclA immunization is not itself protective, boosting with BclA after sub-optimal PA vaccination protected mice against lethal challenge (Brahmbhatt et al. 2007).

While anti-PA antibodies are essential in protection, anti-LF and anti-EF antibodies are contributory and, when combined with anti-PA antibodies, enhance protection. Anti-PA or anti-LF antibodies protect against the cytotoxic effects of LeTx *in vitro* (Paddle et al. 2006).

Although the poly-γ-D-glutamic acid capsule is poorly immunogenic, anti-capsular antibodies have been detected in human cutaneous and oropharyngeal anthrax patients (Sirisanthana et al. 1988) and may contribute to protection against *B. anthracis* infection. Monoclonal antibodies against the capsule can provide significant protection against lethal challenge in a mouse model (Kozel et al. 2004). Immunization with capsule provided some protection against encapsulated, nontoxigenic *B. anthracis* challenge and induced an IgM antibody response, indicating that the capsule is a thymus-independent type 2 (TI-2) antigen, independent of T cell activity. (Wang and Lucas 2004). PGA conjugated to PA as an immunogen stimulated higher antibody titers to both PA and PGA than immunization with either antigen alone, and enhanced survival against live spore challenge (Chabot et al. 2004). Conjugation of PGA to bovine serum albumin (BSA) and keyhole limpet hemocyanin also induced opsonophagocytosis of bacilli and, respectively, enhancement of anticapsule IgG, and antibodies, which bound to the surface of encapsulated *B. anthracis* cells (Schneerson et al. 2003, Wang et al. 2004).

Interspecies Variation in Immune Response to Anthrax

As discussed earlier, laboratory studies indicate that resistance to *B. anthracis* infection and responses to immunization vary between animal species. Anti-PA antibodies demonstrating naturally acquired immunity have been detected in cattle that did not demonstrate illness among herds experiencing anthrax outbreaks. Anti-PA and anti-LF antibodies have been detected in the sera of a variety of herbivorous and carnivorous species in enzootic regions. The relatively low prevalence of antibodies in herbivorous species in the anthrax enzootic Etosha National Park, Namibia, was explained in terms of rapid death in these animals once infected. The high prevalence of antibodies in the relatively resistant carnivores, including vultures, was attributed to the consumption of anthrax carcasses, probably conferring protective immunity and additional resistance on them. Unlike most other carnivores, cheetahs are relatively susceptible to anthrax. This may be attributable to the fact that they do not exhibit scavenging behavior and therefore may not have the opportunity to develop this protective immunity. Naturally acquired antibodies to anthrax are apparently rare in this species. It is not known whether antibody development in the carnivores invariably represents actual infection following scavenging of anthrax carcasses or absorption of incompletely digested toxin and PA. (Relevant references to statements in this paragraph can be found in Good et al. 2008 and Turnbull et al. 2008.)

While there have been no systematic studies of antibody titers with time in vaccinated animals, the evidence is that, after an initial dose, titers fall substantially, if not to undetectable levels within about 3 months. This has been associated with resulting losses due to anthrax. Species differences occur, with at least equines responding poorly to the first dose, but the indications from limited studies are that higher and more lasting titers result after the second and subsequent boosters (Peterson et al. 1993, Turnbull et al. 2004, WHO 2008). This highlights the importance of the use of both priming and booster vaccine doses in immunization programs for susceptible species.

Development of Potential Veterinary Vaccines

In contrast to the extensive recent research targeted at improved vaccines against anthrax in humans there has been little research focusing on improving veterinary vaccines. This is largely attributable to the effectiveness referred to above of the live spore Sterne strain and similar vaccines in reducing the status of anthrax as a global scourge to a relatively minor disease. The *B. anthracis* strains in these vaccines lack plasmid pXO2 and are therefore unable to produce the capsule but still produce the toxin. They thus possess reduced virulence, rather than being totally avirulent, and occasional losses occur, particularly, for reasons as yet unexplained, in goats. Being a live vaccine, generally one dose is sufficient to bring an outbreak under control but, as indicated in the previous section, species differences exist.

The more recent information that exists on the mode of action of the animal vaccines arises largely from attempts to understand why a single dose of live spore vaccine in laboratory animal models gives rise to better protection against challenge with virulent *B. anthracis* than several doses of the human nonliving vaccines. Stimulation of the relevant arms of the cellular and innate immune systems as discussed previously appears to lie at the heart of these differences. However, some further advances have been made, particularly in relation to the residual virulence and the limited duration of immunity they confer. Brossier et al. (1999) developed a modified vaccine strain with a mutant *lef* gene and resulting detoxified lethal factor, which induced anti-PA and anti-LF antibody and protection in mice comparable to the conventional vaccine. In theory, a live spore vaccine formulated with this strain would be wholly safe for any species. Recombinant nontoxigenic, unencapsulated *B. anthracis* (pXO1-/2-) experimental live spore vaccines expressing rPA under control of alternative promoters to *pagA* induced high anti-PA and neutralizing antibody titers and protective immunity of >12 months' duration in guinea pigs (Cohen et al. 2000). In lacking the principal virulence factors of *B. anthracis*, it would be anticipated again that these would be wholly safe for any species.

Plasmid DNA vaccines for use in animals have also been explored. Three doses of plasmid DNA vaccines encoding either PA or the PA domain 4, which mediates contact with the PA receptor on host cells, induced lower but longer lasting anti-PA antibody titers than adjuvanted PA (Hahn et al. 2006). An adenovirus vaccine expressing the PA domain 4 induced IgG in mice 14 days after a single intramuscular injection at levels similar to immunization with the human anthrax vaccine, with titers raised 25- to 30-fold by a booster 4 weeks later (McConnell et al. 2007). Adenovirus-vectored vaccines stimulate potent cellular immunity by promoting high levels of antigen-specific CD4+ and CD8+ cells, and full protection was conferred in the mice within 3 weeks of primary immunization or with the combination of primary and booster immunization.

CONTROL

Control measures at the onset of an outbreak of anthrax consist of identification and elimination of the source of infection; incineration of carcasses; decontamination of the carcass site and of items used for diagnosis; and disposal of the carcasses and commencement of prophylaxis of other animals that are likely to have been exposed to the source of infection. Because sporulation of *B. anthracis* requires oxygen and is therefore inhibited inside an intact carcass, regulations in most countries forbid postmortem examination of animals when anthrax is suspected.

Detailed approaches to these control measures are given elsewhere (WHO 2008) and are beyond the scope of this chapter. In general, if feed is identified as the source of infection, it should be removed and incinerated with appropriate disinfection of items that have been in contact with it. Other animals must be moved away from the affected area and scavengers should be prevented from accessing the carcasses. The affected herd or flock should be quarantined for at least 14 days, preferably 20 days, after the last case. If flies are suspected of being involved, fly control should be initiated. Carcasses should be incinerated. Where shortage of fuel makes this difficult, burial is the less satisfactory alternative; disturbance of former burial sites is too frequently associated with the occurrence of new cases.

Bacillus anthracis is susceptible to penicillin and a wide range of other antibiotics, and animals appear to respond well to treatment even when the infection is in a fairly late stage and showing signs of illness. Such animals may be treated with intravenous penicillin, or penicillin together with streptomycin. Animals suspected of having been exposed to the source of infection may be treated with long-acting penicillin. In enzootic areas, where continuous or regular exposure is likely, annual vaccination is advisable. Generally, treatment is only appropriate for livestock. Vaccination is frequently appropriate for at-risk endangered wildlife species and other species in small game ranches. For obvious reasons, the live vaccine should not be given to an animal being treated with antibiotics. Guidelines on the availability and use of currently licensed animal vaccines against anthrax are given elsewhere (WHO 2008).

FUTURE DIRECTIONS

There remains much to be understood about the life cycle of *B. anthracis* and how this successful pathogen causes disease. The structure and composition of the spore is critical for its survival in the environment and for its ability to infect a host. Future

research will provide additional information on how the spore can remain viable during long periods of dormancy in harsh conditions while able to detect conditions favorable for germination and then transform into a metabolically active cell. The initial interactions between the spore and the host are not well understood and the discovery of potential mechanisms that facilitate infection will be of considerable interest.

Research efforts will continue to focus on virulence factors. The biosynthesis of the capsule has been described only in general terms, and there is a great deal to be learned about the structures and enzymology of this process. Although there has been considerable work on anthrax toxin, there are fundamental questions that need to be answered: Which of the myriad of effects of the toxin are crucial for disease progression? How do animals die from the toxin? There are also a number of other virulence factors that have only recently been reported that deserve attention and the regulation of gene expression of all these virulence factors continues to be an important area of research.

The immune response to *B. anthracis* is essential for controlling the infection. A better understanding of the cell types and processes that limit infection is needed, and studying differences between the immune responses of resistant and sensitive animal species could yield important findings. These and other studies should aid vaccination protocols and control measures.

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7
Mycobacterium

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INTRODUCTION

Mycobacteria belong to the Order *Actinomycetales*, Family *Mycobacteriaceae*. The genus *Mycobacterium* includes the *Mycobacterium tuberculosis* and *Mycobacterium avium* complexes, other pathogenic mycobacteria, and numerous species of saprophytic microorganisms present in soil and water. The *Mycobacterium tuberculosis* complex includes *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. bovis*, *M. pinnipedii*, *M. caprae*, and *M. microti* (fig. 7.1A). The *M. avium* complex includes *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis*, and *M. intracellulare* (fig. 7.1B). Some other mycobacteria of clinical significance are *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium kansasii*, *Mycobacterium leprae*, *Mycobacterium marinum*, *Mycobacterium ulcerans*, and *Mycobacterium scrofulaceum*.

CHARACTERISTICS AND SOURCES OF THE ORGANISMS

Mycobacteria are obligate aerobes, nonspore forming and nonmotile bacilli, and are 0.6–1.0 × 1.0–10 µm in size. Their high cell wall lipid content excludes standard aniline dyes, so that once stained with special staining procedures, mycobacteria are resistant to decolorization even by acid alcohol. This property is termed acid fastness, so that mycobacteria are commonly referred to as acid-fast bacilli. In contrast, these microorganisms are not readily stained with the Gram method and are considered weakly gram-positive. Growth rates for mycobacteria are slow, with generation times ranging from 2 to more than 20 h. Based on different generation times, mycobacteria can be divided into slow and rapid growers. Slow growers require more than 7 days to form visible colonies on solid medium, whereas rapid growers form colonies within 7 days (Holt et al. 1994).

Tuberculosis remains the leading cause of death in humans caused by a single infectious agent, being responsible for nearly 2 million deaths annually. It is caused primarily by *M. tuberculosis*; *M. africanum*, *M. bovis*, and *M. canettii* account for less than 1% of tuberculosis in humans (Thoen and LoBue 2007; Thoen et al. 2009). In addition to the host adaptation of *M. bovis* largely to cattle, other host-adapted variants of *M. bovis* have been designated (Hewinson et al. 2006), such as *M. pinnipedii* (seal-adapted) and *M. caprae* (goat-adapted). Interestingly, *M. bovis* bacille Calmette-Güerin (BCG), attenuated by *in vitro* passages on potato slices, is used to vaccinate humans throughout the world. *M. africanum* and *M. canettii* are human pathogens. *Mycobacterium microti* has been isolated from humans, voles, and some other animals (Thoen et al. 2009). Pathogenic mycobacteria all produce granulomatous lesions in tissues of humans and a wide range of domestic and wild animals. Although the tubercle bacillus was discovered more than 120 years ago, definitive information on its pathogenesis is not yet available, although understanding is developing at a remarkable rate. Unexplained differences in susceptibility of different animals to various acid-fast bacilli occur (Thoen and Barletta 2004). *M. tuberculosis*, the human

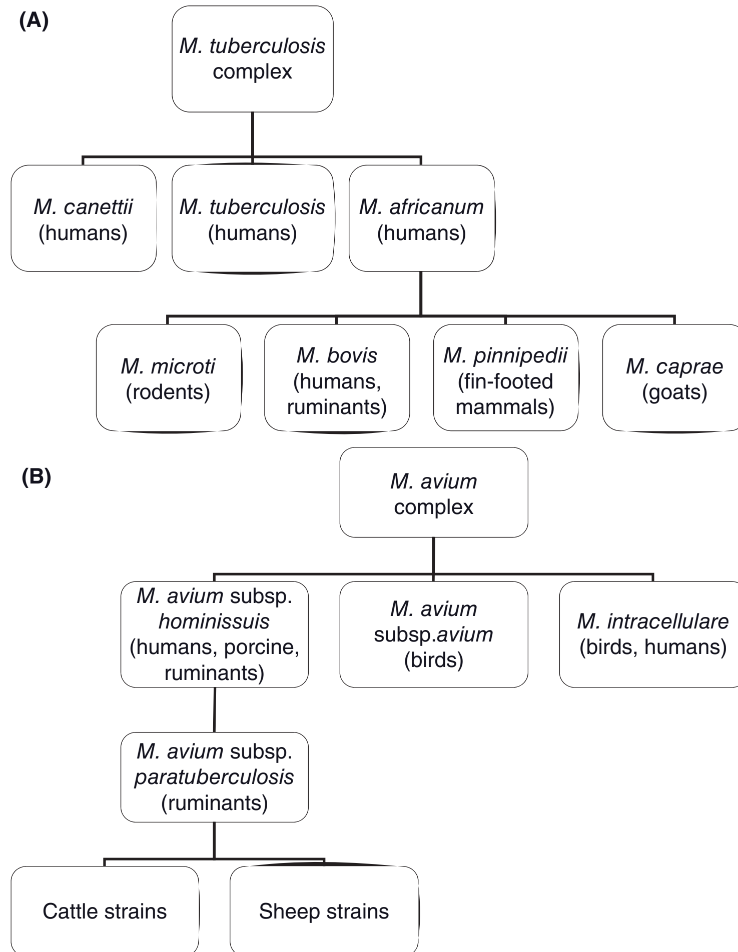

Figure 7.1. Proposed evolution of the *M. tuberculosis* and *M. avium* complexes The tree diagrams for the proposed evolution of members of the *M. tuberculosis* (A) and *M. avium* (B) complexes are shown. See text for further details and citations.

tubercle bacillus, produces progressive generalized disease in nonhuman primates, dogs, swine, and guinea pigs, although cattle and cats are quite resistant (Thoen 2010). *M. tuberculosis* may induce tuberculin skin sensitivity in cattle and other animals. *M. bovis*, the agent of bovine tuberculosis, is a slow-growing nonphotochromogenic organism that also causes disease in other domestic and wild animals, and has been reported in humans in several countries (Thoen et al. 2006; Thoen et al. 2009). Biochemical tests are available for differentiating bacteria of the *M. tuberculosis* complex, but molecular techniques are now widely used in reference laboratories around the globe (Harris 2006; Thoen et al. 2009).

Mycobacterium leprae, the cause of leprosy (also known as Hansen's disease), is a chronic granulomatous disease. Leprosy is still considered a public health problem in countries in Africa and Southeast Asia, with a prevalence rate of more than 1 case per 10,000 individuals (Meima et al. 2004). *M. leprae* and leprosy has been identified in armadillos in the United States. *Mycobacterium lepraemurium* has been isolated from leprosy-like lesions in cats, rats, and mice. *Mycobacterium chelonei, M. intracellulare*, *M. marinum*, *M. nonchromo-*

genicum, and certain other mycobacteria have been isolated from granulomatous lesions in cold-blooded animals.

Microorganisms of the *M. avium* complex have the widest host range among all mycobacteria. *M. avium* subsp. *avium* (serovars 1, 2, and 3) are isolated from tuberculous lesions in humans, birds, domestic, and wild animals (Thoen et al. 1981). In birds, disease is usually progressive, with lesions in the liver and spleen, whereas lesions in other animals are usually confined to lymph nodes associated with the intestinal tract. *M. avium* subsp. *hominissuis* is the subspecies most frequently causing lesions in humans and swine, and *M. intracellulare* is widely distributed in the environment, causing granulomatous lesions mainly in cold-blooded animals (Thoen 2010). Rabbits are highly susceptible to experimental infection with *M. avium* subsp. *avium*, but relatively resistant to *M. intracellulare.* Interestingly, birds are susceptible to *M. avium* subsp. *avium* but resistant to infection by members of the *M. tuberculosis* complex (Thoen and Barletta 2004).

Mycobacterium avium subsp. *paratuberculosis* causes a transmissible intestinal disorder of ruminants commonly known as Johne's disease that has a significant economic impact on the livestock industry (Harris and Barletta 2001). Cattle, sheep, goats, and certain wild ruminants are susceptible. In addition, it has been suggested that this microorganism may be the etiologic agent of Crohn's disease, an inflammatory bowel disease in humans (Chacon et al. 2004). However, this issue still remains controversial. A characteristic that is useful in differentiating this organism is its dependency on mycobactin, an iron-chelating agent, for *in vitro* growth. Mycobactin was initially extracted from *Mycobacterium phlei*, but later mycobactin J and certain extracellular iron-binding compounds were isolated from *M. avium*. Molecular techniques such as PCR and restriction endonuclease analysis have been developed for identifying *M. avium* subsp. *paratuberculosis* (Harris and Barletta 2001).

Mycobacterium ulcerans causes chronic skin ulcers in humans, termed Buruli ulcer or Bairnsdale ulcer. These lesions are caused by the effects of mycolactone, a polyketide-derived macrolide isolated from *M. ulcerans* (George et al. 1999). Studies have pointed to water insects from the family *Naucoridae* as a possible vector for the transmission of *M. ulcerans* (Marsollier et al. 2002). *M. marinum* causes tuberculosis in fish and amphibians as well as cutaneous granulomatous disease in humans, known as swimming pool granuloma.

Other species of mycobacteria have been isolated from various animals (Thoen et al. 1981). *Mycobacterium fortuitum*, a rapid-growing, nonchromogenic organism has been isolated from humans and dogs with lung lesions, cattle with mastitis, and lymph nodes of slaughter cattle and swine. *Mycobacterium chelonae,* also a rapid grower, has been isolated from swine and humans. Granulomatous lesions in swine and cattle, which closely resemble lesions caused by *M. bovis*, have been reportedly caused by *M. kansasii*, a slow-growing, photochromogenic organism.

BACTERIAL VIRULENCE FACTORS

In recent years, remarkable progress has been made in understanding the basis of virulence and the pathogenesis of mycobacterial infections particularly through the application of whole genome sequencing and comparative genomic analysis.

Genomics

The science of genomics has made possible the elucidation of the complete genetic blueprint of several mycobacterial species of importance in human and veterinary medicine, as well as environmental species. Complete genome sequences are now available for *Mycobacterium abscessus*, *M. avium* subsp. *hominissuis, M. avium* subsp. *paratuberculosis*, *M. bovis, M. bovis* BCG, *Mycobacterium gilvum*, *M. leprae*, *M. marinum, Mycobacterium smegmatis*, three strains of *M. tuberculosis*, *M. ulcerans*, *Mycobacterium vanbaalenii*, and four mycobacteria unclassified at the species level These genomes possess high GC content (~65%). Major findings for two mycobacterial species of veterinary importance are reported below.

Sequencing and annotation of the *M. avium* subsp. *paratuberculosis* genome from strain K-10, isolated from a cow with Johne's disease, have been completed (Li et al. 2005). This strain had a low number of *in vitro* passages, and a genetic system including transposon mutagenesis is available for the creation of mutant strains (Foley-Thomas et al. 1995; Harris et al. 1999). The K-10 genome is a circular chromosome of about 4.8 Mb encoding 4344 open reading frames (ORFs) with a 69.3% GC content. About 60% of the ORFs have known homologues in

databases while 25% encode putative proteins of unknown functions. About 75% of the *M. avium* subsp. *paratuberculosis* genes have counterparts in *M. tuberculosis*, and although most genes have orthologs in *M. avium* subsp. *hominissuis*, there are 39 predicted proteins that are unique to *M. avium* subsp. *paratuberculosis.* ORFs are identified by a location number following standard conventions (e.g., *M. avium* subsp. *paratuberculosis* 1152 signifies ORF1152 from the ORF0001—DnaA—in the clockwise direction; see also Wu et al. 2009 for corrections to the original assembly).

The genome possesses high redundancy because of gene duplication, especially for genes involved in lipid and redox metabolism. Nonetheless, differences from other mycobacterial genomes are noted in the low abundance of PE and PPE families in *M. avium* subsp. *paratuberculosis*. In addition, the salicyl-MP ligase gene (*mbtA*) is truncated, which is likely the basis of its defect in mycobactin biosynthesis. Analysis of genetic polymorphisms, especially those including large sequences, indicates that *M. avium* subsp. *paratuberculosis* originated from *M. avium* subsp. *hominissuis* in a biphasic evolutionary process (Alexander et al. 2009). First, an original pathogenic clone of *M. avium* subsp. *paratuberculosis* arose by acquisition of novel DNA and polymorphisms. Second, sheep and cattle strains arose from this ancestral clone by subsequent lineage-specific deletion events.

Genome sequencing demonstrated that the *M. bovis* genome (4 345 492 bp for the virulent bovine isolate AF2122/97) is a down-sized version of the genome of *M. tuberculosis* (4 411 532 bp for the human isolate H37Rv), with more than 99.95% identity and no new genetic material as compared to *M. tuberculosis* (Garnier et al. 2003). Thus, DNA deletions in *M. bovis* are the major contributors to these differences, which have been found to affect genes involved in transport, cell surface structures, and intermediary metabolism. These deletions may remove genes that are unnecessary for host adaptation and lead to a different and sometimes even wider host range. Point mutations also play a role in defining the phenotype, as it is the case for *M. bovis* resistance to pyrazimamide. In addition, sequence variations have been found in genes coding for cell wall and secreted proteins, such as the PE_PGRS and PPE protein families. Another notable change is a mutational event in the *M. bovis* pyruvate kinase gene that renders *M. bovis* unable to use glycerol as a carbon source. Other sequence changes involve master regulatory genes controlling the expression of multiple gene families. The analysis of the *M. bovis* genome challenged the epidemiological hypothesis that *M. tuberculosis* was a human-adapted variety of *M. bovis* that was acquired from cattle. The irreversible loss of DNA material uncovered by the *M. bovis* genome sequencing and the systematic analysis of polymorphisms in a large panel of strains led to the new paradigm that *M. canettii* is likely the ancestral species of the *M. tuberculosis* complex. Successive DNA deletions, starting by the loss of region RD9 (RD stands for regions of difference), led to differentiation of *M. africanum*, *M. bovis*, and *M. microti.* Moreover, *M. bovis* BCG experienced further deletions during *in vitro* laboratory adaptation and its loss of region RD1 has been implicated as the mechanism of virulence attenuation. Thus, similarly to *M. avium* subsp. *paratuberculosis*, evolution of mycobacterial genomes involves a dominant process of reductive deletions. Based on comparative genomic studies, fig. 7.1 depicts an overall tree diagram for the evolution of mycobacterial genomes (Brosch et al. 2002; Devulder et al. 2005; Mueller et al. 2008; Alexander et al. 2009).

Genome sequencing of mycobacteria has been enhanced by parallel developments in the genetic systems used to create defined mutants and to elucidate the function of each gene in the pathophysiology of mycobacterial infections (Braunstein et al. 2002). Both plasmid and mycobacteriophage vectors have been used extensively to create recombinant or mutant strains using all means of genetic exchange, including transformation, transduction, and conjugation. A variety of reporter genes have been expressed in mycobacterial species including beta-galactosidase, firefly luciferase, and the green fluorescent protein. In addition, several vectors for conditional or antisense expression in mycobacterial systems are now available. Mycobacteriophage vectors have been particularly useful in creating defined mutations in many mycobacterial species, including recent application of this technology to *M.* avium subsp. *paratuberculosis*, one of the slowest growing mycobacterial species (Park et al. 2008). Finally, the elegant technology of transposition-site hybridization (TraSH) mutagenesis has been developed to define genes essential under any desired condition (Sassetti et al. 2003; Sassetti and Rubin 2003).

Molecular Strategies to Identify Virulence Determinants

Several strategies are being followed to mine mycobacterial genomes for virulence determinants or antigens of diagnostic importance. One approach seeks to identify *M. avium* subsp. *paratuberculosis* genes whose homologues in other mycobacteria, mostly *M. tuberculosis*, have been flagged as candidate virulence determinants. For example, a tyrosine phosphatase capable of interfering with macrophage activation has been identified as *M. avium* subsp. *paratuberculosis* 1985 whose homologue in *M. tuberculosis* is Rv2232 (Bach et al. 2006). Comparative genomic approaches have identified a set of 39 genes unique to *M. avium* subsp. *paratuberculosis* whose encoded proteins may have diagnostic significance (Bannantine and Paustian 2006). Screening of a *M. avium* subsp. *hominissuis* transposon library led to the identification of a pathogenicity island that carries important virulence determinants involved in bacterial uptake by macrophages (Danelishvili et al. 2007). Comparative genomic hybridizations identified genomic islands in the *M. avium* subsp. *paratuberculosis* genome demonstrating a gene organization different from subsp. *hominissuis* (Wu et al. 2006). Another approach has been the screening of transposon mutant libraries. One such study directly sequenced strain ATCC 19698 transposon mutants and classified them into functional groups (Shin et al. 2006). To ascertain a role in pathogenesis, 11 mutants were selected, based on bioinformatic analysis, and used in mouse virulence experiments. This analysis led to the identification of the following genes: *gcpE*, *pstA*, *kdpC*, *papA2*, *impA*, *umaA1*, or *fabG2_2*. Recent molecular approaches used to identify virulence determinants have involved transcriptomic analyses and TraSH mutagenesis (Talaat et al. 2000; Fisher et al. 2002; Stewart et al. 2002; Sassetti et al 2003; Sassetti and Rubin 2003; Wu et al. 2007; Tailleux et al. 2008)

PATHOGENESIS

Overview

Mycobacteria have evolved as pathogens to survive in macrophages and to overcome multifactorial hostile innate and acquired host immune mechanisms, so that they can survive and eventually multiply and transmit to other animals. As successful pathogens, mycobacteria have developed strategies to turn sets of genes on and off in response to the distinct environments encountered during different stages of infection. First, the microorganisms gain entry into host macrophages, where they survive and replicate intracellularly. For example, mycobacteria use multiple cell surface receptors, including the mannose, complement, and Fc receptors to gain entry into macrophages (Schlesinger et al. 1990; Bartow and McMurray 1998). Subsequently, mycobacteria reside within a membrane-bound vacuole and prevent the maturation of this compartment. This arrest involves a series of alterations in the phagosome, such as changes in the pattern of acquisition of Rab proteins and GTPases, exclusion of the proton ATPase from the phagosome, and retention of a phagosome coat protein that would normally be released from this compartment prior to phagolysosome fusion (Sturgill-Koszycki et al. 1996; Ferrari et al. 1999; Russell et al. 2002; Deretic et al. 2004; Deretic et al. 2006). Macrophages may also use an autophagic process to bypass the blockage on phagosome maturation imposed by *M. tuberculosis* (Deretic 2008).

Pathogenicity of mycobacteria is a multifactorial phenomenon requiring the participation and cumulative effects of several components, including complex lipids and proteins in both the cell wall and the cytoplasm of tubercle bacilli (McNeil and Brennan 1991). Most studies of mycobacterial virulence determinants have been performed with *M. tuberculosis* rather than other mycobacteria. However, the presence of homologous genes in other mycobacteria and the close relationship between these microorganisms suggest the use of similar virulence determinants and mechanisms of pathogenicity.

Role of Complex Carbohydrates and Lipids

The cell wall core of mycobacteria is composed of three covalently attached molecules: peptidoglycan, arabinogalactan, and mycolic acid. Lipids and glycolipid complexes present in the cell wall of virulent and attenuated strains of mycobacteria have been extensively examined to understand their significance in granuloma formation (Ehrt and Schnappinger 2007; Jain et al. 2007). For example, the glycolipid trehalose-6,6′ dimycolate endows *M. tuberculosis* with the ability to form cords when grown in liquid culture medium (Goren et al. 1979; Glickman et al. 2000). Mycobacteria differ in sulfur-containing glycolipids. Sulfolipids and

sulfatides are present in *M. tuberculosis* but absent from *M. bovis* because the glycolipid sulfotransferase and arylsulphatase genes are disrupted in the latter (Rivera-Marrero et al. 2002). This difference may also contribute to determining the host range and tissue tropism of *M. bovis*. Another glycolipid, lipoarabinomannan (LAM), may contribute to arresting phagosome maturation. In addition, LAM is a powerful scavenger of reactive oxygen (ROI) and nitrogen (RNI) intermediates (Chan et al. 1991). Moreover, *M. tuberculosis* and *M. bovis* contain mannosylated LAM (ManLAM), which provides a way for mycobacteria to enter phagocytes via mannose receptors (Schlesinger 1993).

Role of Proteins and Lipoproteins

Mycobacterial proteins and protein complexes (i.e., lipoproteins) also play important and diverse roles in pathogenesis. For example, *M. avium* subsp. *paratuberculosis* attachment and internalization of bacilli to the intestinal mucosa appears to be mediated by FAP-P, a protein of approximately 36 kDa made as a 54-kDa precursor and located in the inner part of the cell envelope (Secott et al. 2001). A recombinant strain carrying a FAP-P antisense construct demonstrated reduced expression and attachment to fibronectin (Secott et al. 2002). In addition, a 35-kDa membrane protein that reacts with antisera from cattle with Johne's disease has been shown to play a role in the invasion of bovine epithelial cells (Bannantine et al. 2003). Proteins encoded by the *mce* operons seem to play a role in the entry and survival of mycobacteria within phagocytic cells, and invasion of epithelial cells (Chitale et al. 2001; Kumar et al. 2003; Gioffre et al. 2005). There are four *mce* operons in *M. tuberculosis*, each encoding 5 to 6 proteins, while the *mce3* operon is absent from *M. bovis* (Cole et al. 1998; Garnier et al. 2003).

The secreted proteins in the antigen 85 complex play an important role in the development of cell-mediated immunity (Andersen et al. 1991). These proteins also possess enzymatic activity and catalyze mycolyltransfer reactions involved in the final stages of mycobacterial cell wall assembly (Belisle et al. 1997). Superoxide dismutases (SODs) are also released into culture supernatant fluids by several mycobacterial pathogens (Harth and Horwitz 1999; Liu et al. 2001). *M. bovis* and *M. tuberculosis* possess a redundant system of SODs. The iron—manganese-dependent SOD (SodA) is secreted and seems the more critical enzyme for resistance against ROI (Edwards et al. 2001). Another membrane–associated copper—zinc-dependent SOD (SodC) may play an additional role in protecting tubercle bacilli against the oxidative burst of activated macrophages (Piddington et al. 2001). Protection against RNI is provided by two alkyl hydroperoxidases denominated AhpC and AhpD (Sherman et al. 1999; Olsen et al. 2000).

Virulence Gene Regulation

Successful infection is dependent on the ability of mycobacteria to adapt to different conditions such as exposure to ROI and RNI, hypoxia, low pH, nutrient starvation, and damage to the cell surface. Mycobacteria thus have several transcriptional regulators that control expression of distinct sets of genes. The best described of these are sigma factors and two-component systems.

Two-component systems consist of a transmembrane sensor histidine kinase that reacts to various stimuli and subsequently activate its transcriptional regulator. These systems have been studied in *M. tuberculosis*, but little is known about them in *M. bovis* and *M. avium* subsp. *paratuberculosis*. Genome sequencing has revealed that conserved two-component systems are present in these species and may have similar functions.

One two-component system that is important for virulence is PhoPR. An *M. tuberculosis* PhoP knockout mutant was more attenuated than BCG and conferred protective immunity against tuberculosis (Martin et al. 2006). In addition, an outbreak of *M. bovis* in humans was caused by a strain that elicited increased expression of PhoP due to insertion of IS*6110* in the promoter region (Soto et al. 2004). Insertion of IS*6110* in the opposite direction and at a different site of the promoter has been demonstrated in some BCG strains (Leung et al. 2008). This may lead to diminished expression of PhoP and may explain some of the attenuation of these strains.

DosRS, or the dormancy regulon, is believed to be relevant for latent infections and is activated during hypoxia and by nitric oxide. It was required for full virulence, but the mutants showed a different attenuation pattern in the various animal models assessed (Converse et al. 2009). Interestingly, while the *M. avium* subsp. *paratuberculosis* DosR has 93% similarity to the *M. tuberculosis/M. bovis* ortholog on the amino acid level, the downstream sensor DosS histidine kinase only has a similarity of

60%. In comparison, a similarity of 80–95% is present between the other sensor histidine kinases of the conserved two-component systems. One could thus speculate that the dormancy genes may be differently regulated in *M. avium* subsp. *paratuberculosis* compared with *M. tuberculosis* and *M. bovis.*

Other two-component systems such as PrrAB and SenX3-RegX3 also appear to play a role in virulence, and MprAB is important for persistent infections. The MprAB also regulates stress responses, including the sigma factors SigB and SigE (He et al. 2006). Sigma factors form a reversible complex with RNA polymerases that provide promoter recognition and thus affect gene transcription. The primary sigma factor SigA and the primary-like SigB are conserved in mycobacteria and allow transcription of housekeeping genes (Manganelli et al. 2004). SigA is essential for growth, whereas SigB may serve as a backup mechanism since the subdomains responsible for promoter recognition are almost identical to those of SigA. SigA may be involved in virulence since a mutant with an amino acid substitution led to attenuated growth of *M. bovis* in guinea pigs. Mycobacteria also have several sigma factors (11 in *M. bovis* and 17 in *M. avium* subsp. *paratuberculosis*) that are responsive to changes in the environment and belong to the group of extracellular function sigma factors (Manganelli et al 2004; Sechi et al. 2007). One of these is SigF, which in *M. bovis* BCG is induced after exposure to cold-shock, hypoxia and oxidative stress. Interestingly, this was not the case in *M. tuberculosis*. This demonstrates that care should be taken when extrapolating results from one mycobacterial species to another, especially when it comes to the complexity of gene regulation. SigE and SigH are also likely to be relevant for virulence since *M. tuberculosis* mutants showed altered virulence either in cell culture or *in vivo* experiments. For the SigH mutant the observed phenotype was rather subtle. No change in bacterial load was observed; however, there were differences in lung pathology with fewer granulomas and a decreased inflammatory response (Kaushal et al. 2002). The sigma factors are part of a tightly regulated network the complexity of which is illustrated by the presence of both anti-sigma factors and of anti-anti-sigma factors. Furthermore, sigma factors also regulate each other, and as already mentioned, can interact with two-component systems.

Lesions

The primary mycobacterial lesion is the granuloma, which is found in affected tissues and the draining lymph nodes. It consists of a core of infected macrophages and macrophage-derived giant cells surrounded by T cells, B cells, neutrophils, and fibrotic tissue. The structure of the granuloma contains the mycobacteria and prevents spread to other areas, but granulomas also provide a niche where the bacteria can hide from the immune system. Although the bacteria can lie quiescent for many years, the granuloma appears to be a dynamic structure where a balanced cytokine milieu seems important to control the infection. In most cases the mycobacteria are contained within the granuloma and the infection is under control, often referred to in humans as "latent TB." Whether latency is a feature of domestic livestock infected with *M. bovis* is still open for debate. Clearly there are animals that are skin-test and/or IFN-γ positive but in which no lesions can be detected, and this might reflect latency. Furthermore, during the Australian tuberculosis eradication program, previously negative animals developed infection years after the herd was declared as tuberculosis free. Such animals can obviously represent a possible source of re-infection, whether they represent true latency with reactivation or a slowly developing chronic infection. The exact mechanisms why some individuals lose control of the primary infectious foci are not clear but are fundamental to understanding mycobacterial infections. Because of the complexity, it is likely that many different forms of immunosuppression can lead to disease progression. This is illustrated by HIV infection in humans where decreased levels of CD4+ T cells result in the reactivation of tuberculosis. The reactivation of tuberculosis encountered in older people also suggests that a less efficient immune response can lead to the loss of control of a dormant infection.

IMMUNITY

The outcome of mycobacterial infections is dependent on a complex interplay between the invading bacteria and the immune responses of the host. The immune responses have been extensively studied in the *M. tuberculosis* complex infections using the murine or guinea pig model. Considerable information has become available, and well-documented theories for the interaction between the bacilli and

the immune system of the infected host have been proposed. Nevertheless, when applying the hypothesis obtained from studies in inbred mice to an outbred human or livestock population, the outcome is more complex. The difficulties in dissecting the roles of individual cell types and cytokines are partly due to the extensive redundancy in the immune system. The many overlapping functions of the components of the immune system are probably a great advantage for the host in the battle against mycobacterial infections, and, indeed, in the majority of infected individuals the host wins the battle. However, it is likely that there is also redundancy and complexity in the bacterial factors involved in virulence, making understanding of the intimate relationship between host and pathogen complex as well as fascinating.

Innate Immunity

Mycobacteria are intracellular bacteria that survive and replicate inside host cells. Both *M. avium* subsp. *paratuberculosis* and *M. bovis* elicit immune responses with similar characteristics. They invade dendritic cells and macrophages, but other phagocytes and tissue cells can also be infected. Such nonprofessional phagocytes and tissue cells may provide a niche for the survival of the bacteria. After ingestion, *M. avium* subsp. *paratuberculosis* is probably transported by specialized M cells residing in the intestinal mucosa through the epithelial barrier where the bacteria are ingested by subepithelial macrophages (Momotani et al. 1988), whereas *M. bovis* is first taken up by alveolar macrophages. Inside the macrophages, mycobacteria prevent the fusion of the lysosome with the phagosome and avoid lysosomal killing mechanisms. The bacteria are able to replicate inside these nonactivated macrophages, eventually leading to the death of the infected cells. The liberated bacteria are phagocytosed by freshly recruited macrophages activated by cytokines such as IFN-γ and TNF-α, which make them better equipped to kill the bacteria. TNF-α has the capacity to exert both beneficial and detrimental effects within the lesions, illustrating the dualism between protective immunity and immunopathology seen in tuberculosis. Lack of TNF-α in mice resulted in loss of granuloma formation and subsequent death (Chakravarty et al. 2008), whereas anti-TNF-α treatment of humans with Crohn's disease gave enhanced susceptibility to tuberculosis (Keane et al. 2001). On the other hand, excess TNF-α can be detrimental, especially when superimposed on lesions with a Th2-biased cytokine pattern (Seah and Rook 2001).

Several other cytokines are also important for controlling the infection. IL-12 is produced by infected macrophages and is necessary for the development of a protective inflammatory response. A major function of IL-12 is to induce IFN-γ and hence tune the specific immune system onto a T helper 1 (Th1) pathway necessary for protective immunity. It thus represents a link between the innate and adaptive immune response. Several studies have demonstrated that antigen-presenting cells (APC) infected with mycobacteria secreted IL-12 and IL-18 (fig. 7.2A). However, there seem to be differences in cytokine production between the various APCs. This is also noted in cattle where bovine dendritic cells infected with virulent *M. bovis* produced IL-12, TNF-α, and little IL-10, whereas macrophages produced TNF-α, IL-10, and little IL-12 (Hope et al. 2004). Cytokines are primarily produced by dendritic cells and macrophages in response to Toll-like receptor (TLR) signaling, and several molecules from the cell wall of mycobacteria have been shown to interact with the TLR.

IL-12 acts in synergy with IL-18 and induces IFN-γ secretion from natural killer (NK) cells, which are large granular lymphocytes belonging to the innate immune system (fig. 7.2A). These cells are believed to be involved in the first-line defense against intracellular infections, and in addition to IFN-γ secretion they exhibit non-major histocompatability complex (MHC) restricted cytotoxic activity in response to mycobacteria (Katz et al. 1990). NK cells are likely to play a role in the initiation of a Th1 pathway and may function as a link between the innate and the adapted immune response. Although they are not believed to be essential for protection, they do seem to contribute to the protective immune response. Human NK cells responded to extracellular BCG with proliferation, IFN-γ production and cytotoxicity (Esin et al. 2004), and optimized CD8+ T cell effector function in response to *M. tuberculosis* (Vankayalapati et al. 2004). Similar results have been reported in cattle where NK cells can restrict intracellular growth of *M. bovis* and produce IFN-γ in response to both infected dendritic cells and macrophages stimulated with mycobacterial proteins like ESAT-6 (Hope et al. 2002; Olsen et al. 2005).

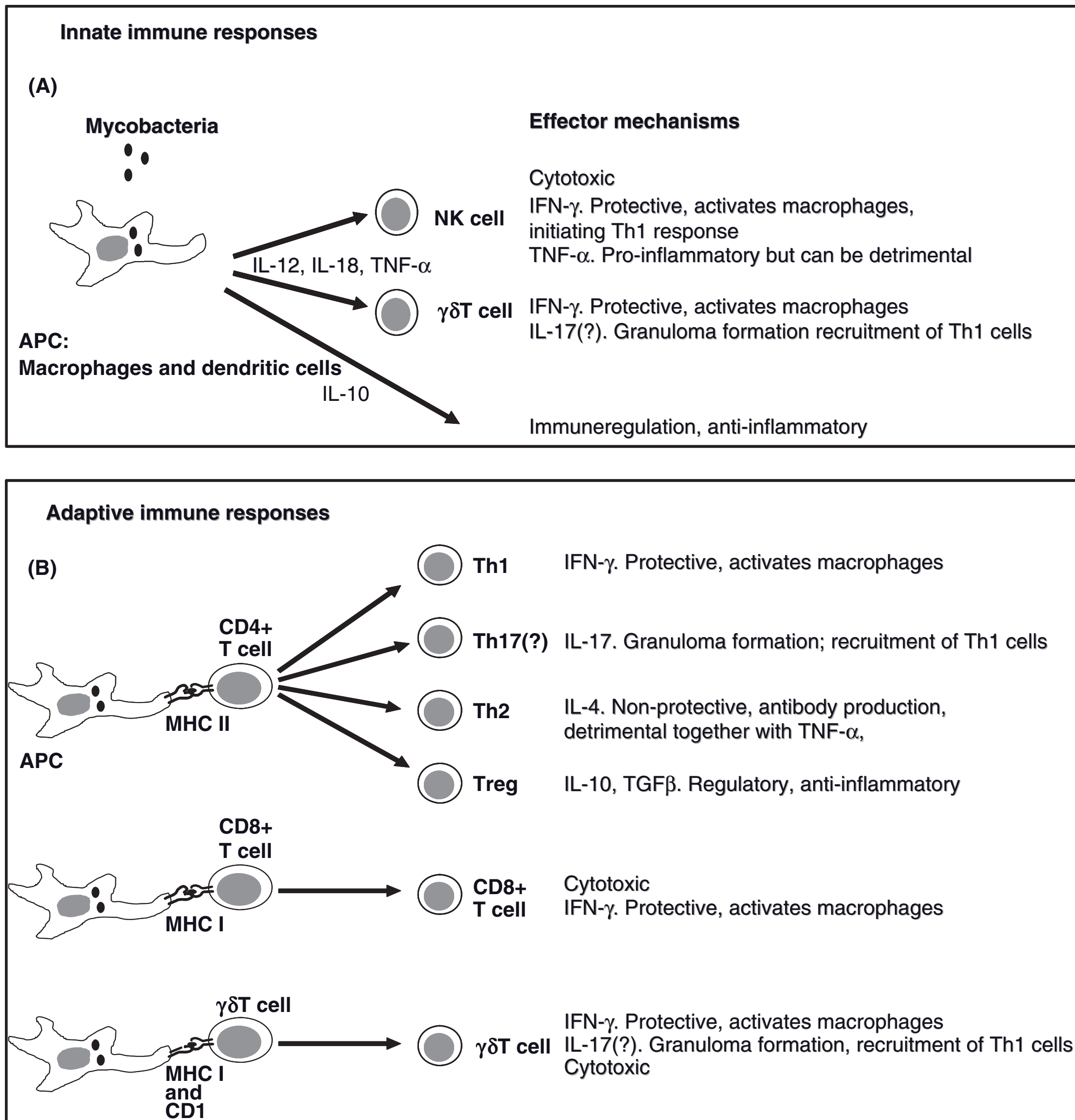

Figure 7.2. Immune responses to mycobacterial infections The figure shows an overview of important innate (A) and adaptive immune responses (B) in mycobacterial infections. CD4+ T cell can develop into different subtypes designated Th1, Th2, Th17, and Treg. To date, Th17 and IL-17 have only been demonstrated in mycobacterial infections in humans and laboratory animals, and this is illustrated by a question mark.

Neutrophils are another cell of the innate immune system that is present in the early mycobacterial lesions. They engulf mycobacteria but seem unable to kill them. After ingestion they may undergo apoptosis, and macrophages and dendritic cells can subsequently engulf these apoptotic bodies. This can lead to enhanced killing mediated by defensins from the neutrophils, but depletion studies have given contradictory results (reviewed in Korbel et al. 2008). The significance of neutrophils in protective immunity or immune-mediated pathology is thus not established.

The innate immune system is clearly important in the first phase of mycobacterial infections but, in general, is not considered sufficient to control them. However, some animals living in heavily infected herds and humans in very close contact with infectious individuals never develop disease or a detectable immune response. These individuals have very likely been exposed to the bacteria, suggesting that the infection was controlled by the innate immune system since activation of the adaptive immune system leads to a positive tuberculin and/or IFN-γ test. Nevertheless, activation of the adaptive immune system is usually considered essential to controlling infection.

Adaptive Immunity

It has long been recognized that the protective immunity against mycobacterial infections is dependent on the activation of the cellular immune defense and that antibodies have little protective value. Among the T cells responding to mycobacteria, the CD4+ αβT cells are the most important. This is illustrated using mice deficient in CD4+ T cells, which demonstrate a much higher susceptibility to mycobacterial infections than mice lacking other subsets of T cells (Leveton et al. 1989). The increased susceptibility to mycobacteria in human AIDS patient is also linked to low CD4+ T cell counts. The CD4+ T cells are present in granulomas and elicit a strong recall response when stimulated with mycobacterial antigens. In granulomatous lesions from cattle with bovine tuberculosis, CD4+ T cells appeared equally distributed in the lymphocytic mantle and in the internal areas of early lesions as opposed to CD8+ T cells that showed a more peripheral localization (Liebana et al. 2007). Similarly in goats with paratuberculosis, CD4+ T cells constituted the main T cell population among the macrophages within the granulomatous lesions (Valheim et al. 2004), whereas a progressive loss of CD4+ T cells was seen in developing lesions in cattle (Koets et al. 2002).

CD4+ T cells react with proteins from the intracellular pathogen that are processed in the endosomal compartment of APCs, with subsequent binding of peptides to the MHC class II molecule. The main mode of action of CD4+ T cells is cytokine production (fig. 7.2B), but human and cattle CD4+ T cells also have cytolytic capacities, a feature in mice that is usually attributed to the CD8+ T cells. The CD4+ T cells were traditionally divided into two counterregulatory subsets referred to as Th1 and Th2, and the balance between these two subsets has been thought to be decisive for the outcome of the infection. The Th1 phenotype produces mainly IL-2, IFN-γ, and TNF-α, and activation of this phenotype is important for protective immunity against mycobacterial infections. The Th2 cells produce cytokines such as IL-4 and IL-5, which induce antibodies and are associated with progressive disease. A key Th1 cytokine, IFN-γ, activates macrophages, which leads to nitric oxide production that can limit growth of mycobacteria. The crucial need for IFN-γ is well established. IFN-γ secretion is dependent on IL-12 produced from macrophages or dendritic cells. The importance of this pathway has been demonstrated in humans where defects in the IL-12 receptor made them more susceptible to infection with mycobacteria. IFN-γ production in both bovine tuberculosis and paratuberculosis has been demonstrated by several authors, and CD4+ T cells were the major contributor (Bassey and Collins 1997; Smyth et al. 2001). In progressive disease, both Th1 and Th2 responses are detected, but what leads to the development of Th2-biased lesions and the development of progressive disease is unclear. Several explanations have been put forward, such as increased antigen load and selective apoptosis of Th1 cells (Seah and Rook 2001).

An alternative explanation is that a shift toward a more Th2-dominated profile is the consequence, rather than the cause, of progressive disease and increased bacterial load. An important Th2 cytokine, IL-4, is increased in both humans and animals with progressive disease (Buddle et al. 2005). Moreover, IL-4 has been shown to lead to increased toxicity of TNF-α, and IL-4 suppressed nitric oxide generation by bovine macrophages that are necessary for controlling intracellular mycobacteria (Jungi et al. 1997). In addition, a splice variant of IL-4 that functions as an antagonist to IL-4 has been described in both humans and cattle and has been suggested to be a marker for protective immunity (Rhodes et al. 2007). These findings suggest that increased IL-4 is relevant for disease progression and not just a consequence of increased bacterial load.

Type 2 (Th2) cytokines also induce antibody production. Antibodies against mycobacteria are, in general, associated with progressive disease and seem to play a minor role for protective immunity. However, passive transfer of antibodies against

LAM conferred partial protection against tuberculosis in mice (Teitelbaum et al. 1998). Such antibodies may influence the uptake of mycobacteria and stimulation of macrophages with subsequent modulation of the immune responses. Some protective effect of antibodies with certain specificities should therefore not be disregarded.

With the recognition of Th17 cells, it has become clear that the CD4+ T cell responses to tuberculosis is more complex than captured by the Th1–Th2 paradigm of immunity. IL-17 was initially described as important in several autoimmune diseases and extracellular infections, but cells that produce IL-17 have also been described in both mice and humans with tuberculosis (Scriba et al. 2008). In mice, there appears to be a distinct lineage of CD4+ αβT cells called Th17 cells, whereas in humans there are often CD4+ T cells with a mixed Th1/Th17 profile. Several cytokines such as TGF-β, IL-6, and IL-23 are involved in the differentiation of naïve T cells into Th17 and/or maintaining IL-17 production from memory Th17 cells. IL-23 is a dimer that shares the p40 subunit with IL-12, implying that previous studies blocking the IL-12 p40 subunit must be reevaluated in the light of the IL-23/IL-17 pathway. The role of IL-23/IL-17 in mycobacterial diseases is still elusive, but it does not appear to be necessary for protective immunity against tuberculosis in mice. However, IL-17-producing cells that are reactive to mycobacterial antigens have been described, and depletion of IL-17 leads to decreased recruitment of IFN-γ producing Th1 cells in the early stages of infection (Khader et al. 2007). It is possible that the cross-regulatory properties of IL-12/IFN-γ and IL-23/IL-17 may be crucial to achieving the right balance between protection and immunopathology. Bovine IL-17 has been identified, but its role in mycobacterial infections remains to be established (Riollet et al. 2006).

The major role of CD4+ T cells in the protective immune response against mycobacteria is undisputable, but the importance of CD8+ MHC class I restricted αβT cells has also been recognized. Early mouse studies using CD8 depletion or adoptive transfer of CD8+ T cells showed somewhat conflicting evidence, but some protective effect of CD8+ T cells has been detected. However, one study depleting CD8+ T cells in the bovine model led to decreased IFN-γ production but diminished pathology, suggesting a role for this cell type in immunopathology (Villarreal-Ramos et al. 2003). The CD8+ T cells recognize peptide antigens presented to their T cell receptor by classical MHC class I molecules. The importance of a functional MHC class I presenting pathway has been demonstrated in both β_2-microglobulin and tapasin (TAP) knockout mice, which were more susceptible to intravenous *M. tuberculosis* infection than their wild-type counterparts. Following aerosol challenge of mice with *M. tuberculosis,* CD8+ T cells trafficked to the lung as early as 2 weeks post infection, and CD8+ T cells are also present in bovine tuberculosis and paratuberculosis lesions (Begara-McGorum et al. 1998; Liebana et al. 2007).

The function of CD8+ T cells is, in general, lysis of infected target cells. Cytolysis mediated by CD8+ T cells involves the release of granules containing perforin and granzyme, or cytolysis can be mediated through the Fas/Fas ligand molecules that result in programmed cell death of the target cell. Cytolytic responses in bovine tuberculosis have been demonstrated (Skinner et al. 2003b). Furthermore, several studies have shown that while CD4+ T cells are the major IFN-γ producer in both bovine tuberculosis and paratuberculosis, CD8+ T cells can also elicit pronounced secretion of IFN-γ (Bassey and Collins 1997; Liebana et al. 1999). There is thus a redundancy in the functions of CD4+ and CD8+ T cells in that both cell-types have cytolytic capacities and can secrete cytokines (fig. 7.2B).

Cattle have a large proportion of T cells that use the γδ T cell receptor instead of the αβ chains. γδT cells constitute 30–80% of the T cells in peripheral blood, with the largest proportion in young calves. This is vastly different from mice and humans, where less than 10% of the peripheral blood lymphocytes are of this subset. It is therefore not unlikely that these cells are more important for resistance to mycobacterial disease in ruminants compared with other hosts. Subsets of γδT cells in ruminants can be distinguished based on the surface marker WC1, and the prominent population in blood expresses WC1 but is negative for CD2, CD4, and CD8 (MacHugh et al. 1997). Another γδT population is negative for WC1 but expresses CD2 together with CD8, suggesting that these cells may be activated via MHC class I. The latter population was found in large numbers in the spleen and intestines. It has also been demonstrated that the ruminant γδT cells express exceptionally diverse antigen receptors, and they have therefore been suggested to have a broader capacity to recognize diverse ligands than

their counterparts in other species (Hein and Dudler 1997). In mice, γδT cells have been shown to expand in the first phase of tuberculosis and have been suggested as a first-line defense against mycobacterial infections, but these cells do not seem to be essential for resistance against tuberculosis (Ladel et al. 1995). However, they may play a role in granuloma formation (fig. 7.2B). In both cattle and humans, they accumulate early in lesion development, but depletion studies in cattle did not markedly change the course of disease (Kennedy et al. 2002).

The γδT cells are able to respond in both an innate and an adaptive fashion. They recognize both protein and nonprotein antigens in the context of classical MHC class I or CD1 respectively, but this cell type can also respond to self-derived stress molecules as well as soluble factors induced by mycobacterial antigens without the need for previous sensitization. Like αβT cells, γδT cells can secrete IFN-γ and have cytolytic capacities. They were able to directly kill both intracellular and extracellular mycobacteria through a granulysin-dependent mechanism (Dieli et al. 2001). Innate IFN-γ production by bovine γδT cells in response to mycobacterial cell wall antigens has also been detected, which was probably dependent on monocytes-derived cytokines (Vesosky et al. 2004). This was in accordance with another study that showed that bovine WC1+ γδT cells were synergistically stimulated by IL-12 and IL-18 to secrete large quantities of IFN-γ (Price et al. 2007).

A memory-type response has also been described with response to protein antigens whereas other studies did not find significant IFN-γ production in response to mycobacterial antigens in γδT cells (Bassey and Collins 1997; Smyth et al. 2001). In addition to IFN-γ it has been demonstrated that γδT cells from tuberculosis-infected mice can secrete IL-17 in an innate fashion (Lockhart et al. 2006). This finding sheds light on results from the *M. bovis* model where γδT cells were recruited to the lungs early after infection. It is possible that innate IL-17, in addition to IFN-γ production from γδT cells, plays a role in the early events of bovine tuberculosis.

Immune Regulatory Mechanisms

Protective immunity to mycobacterial infections is dependent on a strong Th1 response; however, without control, this response can also cause immune-mediated pathology with excessive inflammation. The right balance between protective immunity and immunopathology is thus essential. Several immunoregulatory mechanisms have been described, and both cytokines IL-10 and TGF-β have been shown to dampen the Th1 response. It is still not clear which cells are involved in the regulation of immune responses in mycobacterial infections in ruminants. In mice and humans, a current major topic of research is the reawakening of T suppressor cells, now designated regulatory T cells (Treg). The most studied of these Treg cells are CD4+, CD25+, which express the transcription factor FOXp3. Treg cells have shown to be increased in humans with active tuberculosis, which may suggest that down-regulation of Th1 responses by Treg cells is responsible for reactivation of disease (Guyot-Revol et al. 2006). However, mice depleted of Treg cells did not have increased bacillary loads in a mouse model (Quinn et al. 2006). An immunosuppressive role of IL-10 has been demonstrated in both bovine tuberculosis and paratuberculosis, but a definite role of Treg cells was not established since the source of IL-10 production was not identified (Buza et al. 2004; Denis et al. 2007). In goats naturally infected with paratuberculosis, monocytes were the major producers of IL-10 in peripheral blood, whereas little IL-10 was produced by classical CD4+ and CD25+ Treg cells (Lybeck et al. 2009). Similarly, only γδT cells and monocytes expressed IL-10 and showed regulatory properties using sorted bovine blood cell subsets in a coculture suppression assay (Hoek et al. 2008). Furthermore, regulatory properties of γδT cells were also detected in bovine tuberculosis (Kennedy et al 2002). This does not exclude a role for Treg cells in mycobacterial infections in ruminants, but the relative importance of these cells is not yet established.

Immunity and Disease Control

Mycobacterial diseases are slowly progressive infections with a large proportion of subclinically infected animals. Identification of all these animals is difficult with available diagnostic assays. Furthermore, the bacteria can survive for an extended time in the environment and several species of wildlife can be infected. These factors make bovine tuberculosis and paratuberculosis challenging diseases to control. Most developed countries have control programs for bovine tuberculosis due to the zoonotic risk of *M. bovis*. These programs are usually based on test-and-slaughter regimes with restriction of movement of animals from infected

herds. The most widely used test is the intradermal skin test, in which purified protein derivative (PPD) from *M. bovis* is injected intracutaneously and measures an increase in skin thickness as an index of infection. Despite these control programs, several countries have failed to eradicate bovine tuberculosis. This is particularly true for countries that have a wildlife reservoir, such as the United Kingdom, Ireland, and New Zealand.

Mycobacterium bovis and *M. avium* subsp. *paratuberculosis* can infect a wide range of domestic and wild animals. To what extent the wildlife constitutes a threat to the domestic animals depends on the ability of the species to act as a maintenance or as a spillover host. In the former, the infection can persist by intraspecies transmission alone, whereas in the latter, infection will not persist indefinitely unless there is reinfection from another host. To control the infection in domestic livestock, it is important to focus on the wildlife that can act as maintenance hosts with less emphasis on spillover hosts. Species that are important for the spread of bovine tuberculosis are the badger in the United Kingdom and Ireland, the brush-tail possum in New Zealand, and the white-tail deer in Michigan. *M. avium* subsp. *paratuberculosis* has also been isolated from a wide range of species and most frequently from deer and rabbits. Paratuberculosis in farmed deer and in deer reserves is prevalent in some areas, and it seems clear that deer can maintain the infection in high-density populations (Balseiro et al. 2008). However, the significance of wild deer and other wildlife for the continuation or spread of paratuberculosis in livestock is not clear.

An alternative strategy to wildlife depopulation in reducing the spread of tuberculosis to livestock is wildlife vaccination. To date, injectable vaccines give the best protective immunity but are not a realistic alternative for purposes other than research trials. BCG, the human tuberculosis vaccine, delivered in a lipid formulation as an oral vaccine, has been shown to reduce the severity of disease in an aerosol challenge model (Aldwell et al. 2003). The partial response achieved may lead to a reduced excretion of bacteria and hence diminished spread of infection to livestock. Vaccination of wildlife, together with a focus on improved management practices as a supplement to current control programs, might reduce the transmission of bovine tuberculosis sufficiently to control tuberculosis in livestock.

Another important cause for spread of mycobacterial infections is movement and trading of animals. The long incubation time and slow progression of disease, together with suboptimal tests, makes it difficult to identify all infected animals. As a consequence, there will always be a risk when purchasing animals from a previously infected herd. It has been difficult to assess how much of the spread is caused by animal movement compared with infection from wildlife and survival of bacteria in the environment, but discussion of this aspect, like that of management factors in controlling tuberculosis and paratuberculosis, is beyond the scope of this book.

Vaccination and Immune Testing

Vaccination against mycobacterial diseases can reduce clinical signs and limit the spread of bacteria but is not sufficient to eliminate the infection. For paratuberculosis, both commercial and in-house live attenuated and inactivated whole cell vaccines have been used. Since vaccination interferes with diagnostic tests for paratuberculosis and also bovine tuberculosis, it cannot be used in countries that have control programs for bovine tuberculosis. Vaccines against bovine tuberculosis are currently not used for the same reason. The use of sophisticated vaccines and diagnostic tests may circumvent this problem in the future. A strategy is to use different antigens in the vaccines and the diagnostic tests, a strategy denominated DIVA (Differentiation between Infected and Vaccinated Animals) strategy. This is already feasible for bovine tuberculosis with the traditional BCG vaccine. *Mycobacterium bovis* BCG lacks several genetic regions compared with virulent *M. bovis*, including the highly immunogenic ESAT-6 protein. A test measuring IFN-γ after *in vitro* stimulation with ESAT-6 can be used to distinguish between infected and BCG-vaccinated animals (Buddle et al. 1999). However, before an IFN-γ test can be approved, comparable sensitivity and specificity to the tuberculin skin test must be documented. To date, this test gives a lower specificity, particularly in young animals, where false-positive results have been reported to be caused by IFN-γ production from NK cells (Olsen et al. 2005). This is of concern since the BCG vaccine gives better protection when given just after birth (Buddle et al. 2003), and it is thus important to be able to test these young animals. An IFN-γ test using specific antigens such as ESAT-6 could also

differentiate between *M. bovis* infected-animals and paratuberculosis-vaccinated animals and thereby allow paratuberculosis vaccination also in areas with bovine tuberculosis.

In addition to interfering with the skin test, the protective effect of BCG in cattle, as in humans, is variable. Although reduced lesions may be observed, the animals are still likely to be infected and a possible source of further spread. Despite substantial effort, it has been challenging to develop second-generation vaccines that give a superior effect to that of BCG. The reason for this is likely that protective immunity is dependent on a complex interaction between various immune cells with a range of anti-mycobacterial effector mechanisms. Several DNA vaccines and subunit protein vaccines have been tested in cattle, but used alone they generally showed lower efficacy than BCG. The most promising alternative is thus BCG combined with certain subunit vaccines, which have been reported to give enhanced protection compared with BCG (Skinner et al. 2003a; Wedlock et al. 2008). These results show that a rational vaccine strategy seems feasible in the future but needs to be accompanied with the development of appropriate diagnostic tests.

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Considerable progress has been achieved by the application of the new molecular genetics and genomics methods, but there are still many issues remaining to be understood in mycobacterial pathogenesis. It is thus not surprising that effective vaccines are still in a developmental phase, with only a few reaching the final testing stages, most of them in the human tuberculosis field. As discussed, the situation is further complicated in animal health as vaccines should not only protect animals but also avoid undesirable cross-species reactions.

Part of this challenge arises from the multifactorial nature of each step in the pathogenic process. Adhesion and invasion may involve a plethora of host cell receptors–microbial protein interactions. An array of antigens, whose expression requires specific timing and tissue expression, participate in the stimulation of the immune system. Mycobacteria possess redundant metabolic routes used to take maximum advantage of tissue microenvironments. Thus, in many experimental studies, a single mutation may not result in a discernible reduction in virulence, thus requiring the construction of multiple knockouts. Complications then arise in the use of different selection markers or with strategies to create unmarked mutations. In some cases, different animal models (e.g., mice vs. guinea pigs) have led to different results regarding the virulence of mutant strains.

Challenges also arise at the interface between the new technologies and the classic acquired knowledge on the biology of these microorganisms. In many cases, this has resulted in the use of attenuated laboratory strains that have already lost the expression of key virulence determinants because of mutations accumulated during *in vitro* passage. In other cases, contradictory findings reported in the literature may be tracked to differences in cultural conditions (media, growth phase, use of detergents to prevent clumping), insufficient analysis of the correlation between phenotypic and genotypic characterization of the strains used in each study (e.g., serotyping vs. spoligotyping), vaccine formulation, routes of inoculation, and so on. Continued progress will require close attention and standardization of these parameters to better correlate the various exciting new studies with known paradigms on the biology of these microorganisms.

Although much is known about protective immunity to mycobacteria, it is still not clear what mechanisms need to be enhanced to get a better efficacy for new vaccines. Should a new vaccine generate stronger Th1 responses? Is it important to have Th1 response to a wider repertoire of antigens? Will a stronger CD8+ response with enhanced cytotoxicity give increased protection? Or is it stimulated Treg cells that can dampen a Th2 response? Perhaps all of the above is necessary to give the optimal result. The best alternative may be to develop a new live attenuated vaccine by deletion of genes from *M. tuberculosis* or *M. bovis*. Such a new attenuated strain of *M. bovis* appeared to provide greater protection than BCG in cattle experimentally infected with *M. bovis* (Buddle et al. 2002). The fear that reversion to virulence by gene acquisition *in vivo* can occur makes it unlikely that such a vaccine will be approved in the near future. In humans, the ability of BCG to protect children from serious forms of the disease such as disseminated tuberculosis makes it ethically difficult to defend a withdrawal of the BCG vaccination program in developing countries. It is thus likely that a boost of the BCG vaccine or a postinfection vaccine will be the future in the human field rather than a replacement.

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8
Corynebacterium and *Arcanobacterium*

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CORYNEBACTERIUM

Corynebacterium species are nonmotile, nonsporulating, short, pleomorphic, gram-positive rods with a high G + C content in DNA. They belong to the class Actinobacteria and are part of the larger *Corynebacterium*, *Mycobacterium*, *Nocardia* (CMN) grouping. Bacteria of this group are characterized by cell walls containing long-chain fatty acids called mycolic acids; corynebacterial mycolic acids have relatively short chains, typically of 28–40 carbons. *Corynebacterium* species are found in a wide range of ecological niches, such as soil, sewage, and plant surfaces, and some are important pathogens of humans or animals.

The *Corynebacterium* spp. that infect animals and have some role in disease production comprise a lengthy list (table 8.1). Other species, including *Corynebacterium aquilae, Corynebacterium capitovis, Corynebacterium caspium, Corynebacterium ciconiae, Corynebacterium falsenii, Corynebacterium felinum, Corynebacterium phocae, Corynebacterium sphenisci, Corynebacterium spheniscorum*, and *Corynebacterium testudinoris*, have been isolated from animals but without any clear connection to disease.

CORYNEBACTERIUM PSEUDOTUBERCULOSIS

Corynebacterium pseudotuberculosis is the corynebacterial species most often seen in animal infections; it also has the greatest economic impact. *C. pseudotuberculosis* is a facultative intracellular pathogen that mainly affects small ruminants, such as sheep and goats, causing a disease known as caseous lymphadenitis (CLA) or cheesy gland. CLA occurs throughout the world but is of most concern in large sheep-producing areas, such as Australia, New Zealand, South Africa, and the American continents. *C. pseudotuberculosis* infection can lead to decreased wool and meat production and increased culling of sick animals and condemnation of carcasses and skins in slaughterhouses (Williamson 2001; Paton et al. 2003; Dorella et al. 2006b). In the Americas, a similar disease occurs in farmed llamas and alpacas (Anderson et al. 2004; Braga et al. 2006). *C. pseudotuberculosis* often causes equine ulcerative lymphangitis, characterized by inflammation of the subcutaneous lymphatic vessels of the lower limbs. Ventral abscessation (also called pigeon fever, pigeon breast, or breastbone fever), folliculitis and furunculosis, and, rarely, mastitis and abortion are other manifestations of *C. pseudotuberculosis* infection in horses (Addo et al. 1974; Rumbaugh et al. 1978; Miers and Ley 1980; Poonacha and Donahue 1995). The organism can cause deep subcutaneous abscessation in cattle, as well as mastitis and necrotic-ulcerative dermatitis of the heel (Yeruham et al. 1997; Kariuki and Poulton 1982; Yeruham et al. 2003a, 2003b). Reports of human infections are rare; however, analysis of 12 cases revealed two that could be linked to occupational exposure, suggesting that a strong case could be made for human zoonotic *C. pseudotuberculosis* infection (Peel et al. 1997).

Classification of *C. pseudotuberculosis* was originally based on morphological and biochemical features, but chemotaxonomy revealed meso-

Table 8.1. *Corynebacterium* spp. Infections in Animal Hosts

Corynebacterium spp.	Major host	Disease	Other hosts	Other diseases
amycolatum	Human	Bacteremia, endocarditis, septic arthritis	Cattle	Mastitis
auriscanis	Dog	Otitis externa	Human	Wound infection
bovis	Cattle	Mastitis	Human, rabbit, hairless mouse	Abscesses, hyperkeratotic dermatitis
camporealensis	Sheep	Mastitis	–[a]	–
cystitidis	Cattle	Cystitis, pyelonephritis	–	–
felinum	Scottish wild cat	Unknown	–	–
glucuronolyticum	Human, pig	Genitourinary tract infection	–	–
jeikeium	Human	endocarditis, bacteremia, septicemia, meningitis, osteomyelitis	Cat	Urinary tract infection
kutscheri	Laboratory rodents	Abscesses, pneumonia, pseudotuberculosis, asymptomatic colonization	Human, vole	Septic arthritis
mastiditis	Sheep	Mastitis	–	–
matruchotii	Horse	Cystitis	–	–
minutissimum	Human	Mixed infection of skin	Cattle	Mastitis
pilosum	Cattle	Cystitis, pyelonephritis	Human, horse	Endocarditis
pseudotuberculosis	Sheep, goat, horse	Caseous lymphadenitis, pericarditis, pleuritis, cellulitis	Human, alpaca, cattle, camel, deer, pig	Lymphadenitis, abscesses, mastitis
renale	Cattle	Cystitis, pyelonephritis	Sheep, goat, deer, laboratory rodents	Cystitis, pyelonephritis, osteomyelitis
suicordis	Pig	Pericarditis		
ulcerans	Human	Pharyngitis, sinusitis, skin infections	Primates, camel, cat, cattle, otter, squirrel	Respiratory infections, caseous lymphadenitis, mastitis
urealyticum	Human	Cystitis, pyelonephritis	Dog, cat	
xerosis	Pig	Arthtitis, abscesses	Goat	Paratuberculosis

[a] No other hosts or diseases have been reported.

diaminopimelic acid in its cell wall. Peptidoglycan, arabinose, and galactose are the major cell wall sugars, and short-chain mycolic acids (corynomycolic acids, 22–36 carbon atoms in length) are also present (Jones and Collins 1986). Other characteristics used to distinguish *C. pseudotuberculosis* from related species *Corynebacterium ulcerans* and *Corynebacterium diphtheriae* are production of phospholipase D (PLD) by *C. pseudotuberculosis* and *C. ulcerans* and production of diphtheria toxin by *C. diphtheriae* and some *C. ulcerans* strains (Barksdale et al. 1981; Groman et al. 1984; Buck et al. 1985; Cianciotto and Groman 1985; Cianciotto et al. 1986).

Results of biochemical testing and restriction endonuclease analysis of *C. pseudotuberculosis* chromosomal DNA led to recognition of distinct biovars *equi* and *ovis* (Songer et al. 1988). This finding was supported by analysis of 16S ribosomal DNA (Vaneechoutte et al. 1995; Sutherland et al. 1996; Costa et al. 1998). Phylogenetic analyses revealed that *C. pseudotuberculosis* and *C. ulcerans* belong to a monophyletic group (Riegel et al. 1995; Hou et al. 1997; Takahashi et al. 1997), but that biovars *equi* and *ovis* should not be classified as subspecies due to their high genomic similarity (Riegel et al. 1995). Use of partial gene sequences from the RNA polymerase β subunit-encoding gene (*rpoB)* has also proved useful in identification and classification of *Corynebacterium* species.

Pathogenesis

CLA is a chronic, granulomatous disease of sheep and goats, characterized primarily by the formation of abscesses within superficial lymph nodes and those draining the lungs. Transmission occurs mainly through contamination of superficial wounds (during shearing, castration, ear tagging, or other traumatic events) by exudate from superficial abscesses or via aerosols from lung lesions (Paton et al. 1995; Williamson 2001). The hypothesis of a respiratory route of infection (Stoops et al. 1984) was based on the observation that some naturally infected sheep have pulmonary lesions only and that some of these lesions are located on airway walls. It has also been possible to produce pulmonary disease by intratracheal administration of bacteria (Brown and Olander 1987). However, lung lesions have also been produced in sheep via intravenous inoculation (Brogden et al. 1984), so the importance of a direct respiratory route of infection remains unclear.

There is evidence of transmission by houseflies and by other Diptera in cattle, buffalo, and horses, although the mechanisms are not well documented (Yeruham et al. 1996; Selim 2001; Spier et al. 2004; Yeruham et al. 2004). Lesions rarely form at the site of entry, but the bacteria multiply and activate an inflammatory response. The bacteria are rapidly transported, generally via the lymphatic system, to proximal draining lymph nodes. There is an initial rapid accumulation of polymorphonuclear leukocytes at the entry point and in the draining lymph nodes, and this is followed within a few days by an increasing recruitment of macrophages (Guilloteau et al. 1990; Pepin et al. 1994). Intramacrophage survival is critical to the trafficking of *C. pseudotuberculosis* from the site of infection to superficial lymph nodes to internal lymph nodes and other reticuloendothelial tissues, where it produces characteristic caseous lesions (Batey 1986; Jolly 1966). Once established in the host, *C. pseudotuberculosis* evades the immune system with relative ease, causing chronic infections that persist for the life of the animal (Baird and Fontaine 2007).

Multiple microscopic pyogranulomas forming in the lymph node grow in size and coalesce to form larger abscesses (Pepin et al. 1991). This may be followed by further extension via the blood or the lymphatics, leading to production of similar lesions in other organs. Viable bacteria may be recovered from abscesses for years after initial infection, and latent disease may be reactivated in the face of appropriate stimuli.

Virulence Factors

Intra-host spread of *C. pseudotuberculosis* depends on PLD production (Carne and Onon 1978). This potent exotoxin has vasodilatory properties that wash the bacteria through the lymphatic system (McNamara et al. 1995; Songer 1997; Baird and Fontaine 2007). PLD also contributes to virulence via dermonecrosis, phagocyte toxicity, complement-dependent lysis of autologous erythrocytes, and lethality at higher doses (Egen et al. 1989; Songer 1997; Tambourgi et al. 2002). Activities of PLD commonly used for identification of *C. pseudotuberculosis* are synergistic hemolysis with *Rhodococcus equi* cholesterol oxidase and inhibition of the staphylococcal β-hemolytic activity (Baird and Fontaine 2007).

Murine or caprine macrophages may be damaged or destroyed due to PLD action. Infection of caprine macrophages resulted in vesiculation of the cytoplasm, mitochondrial damage, dilation of the endoplasmic reticulum, and death less than 20 h after infection (Tashjian and Campbell 1983). Intracellular *C. pseudotuberculosis* has a small but significant effect on macrophage survival (McKean et al. 2007a).

Infection studies with *pld* deletion mutants demonstrated that PLD is essential for full virulence in sheep and goats (Hodgson et al. 1992; McNamara et al. 1994). Site-specific mutagenesis revealed that the His20 residue of PLD is part of the enzyme active site (Haynes et al. 1992). Comparison of *pld* sequences from strains of biovars *equi* and *ovis* revealed several amino acid differences between the predicted proteins; it is not clear whether this is a consistent difference distinguishing the biovars, as genes from only a single isolate of each were sequenced (McNamara et al. 1995). *pld* is down-regulated during heat shock (43°C) but is up-regulated in a cell-density-dependent manner and is highly expressed in infected macrophages (McKean et al. 2007a, 2007b).

Cell wall lipids of *C. pseudotuberculosis* have been considered virulence factors since their cytotoxicity was demonstrated more than 50 years ago (Carne et al. 1956). They protect the bacteria from intraphagocytic destruction but also induce hemorrhagic necrosis and chronic abscessation (Jolly 1966; Hard 1975; Muckle and Gyles 1983; Tashjian and Campbell 1983).

Iron acquisition is vital for *C. pseudotuberculosis*; the *fagBCD* operon, which encodes a putative iron uptake system, has been proposed as a virulence factor. There was no alteration in utilization of iron by a *fagB*(C) mutant *in vitro*, but this mutant had decreased ability to induce abscessation in goats (Billington et al. 2002).

Recent studies used whole genome screening methods to identify genes possibly involved in virulence. *C. pseudotuberculosis* genes expressed during infection of macrophages were identified via a differential fluorescence induction reporter system (McKean et al. 2005). Random transposon mutagenesis with the TnFuZ system identified *C. pseudotuberculosis* genes encoding exported proteins, which are being evaluated for roles in virulence (Dorella et al. 2006a).

Immunity and Vaccination

Primary immune responses to *C. pseudotuberculosis* infection are complex and are mediated by cellular interactions and cytokines. Secondary responses occur through multiple pathways (Ellis et al. 1990; Pepin et al. 1997; Paule et al. 2003). Intact major histocompatibility complex (MHC) class II staining macrophages and various subpopulations of lymphocytes, organized in three distinct regions, have been identified inside encapsulated lesions, and there is evidence that continual lymphocyte recruitment is a feature of chronic CLA (Walker et al. 1991). Interferon-gamma (IFN-γ) and complement receptor 3 (CR3) play important roles in control of primary *C. pseudotuberculosis* infections in mice (Simmons et al. 1997; Lan et al. 1999a) and IFN-γ, CR3, and tumor necrosis factor alpha TNF-α are important in the host response to secondary infections (Lan et al. 1999a, 1999b).

Commercial vaccines are available in various regions of the world. Combined vaccines of the Glanvac™ series, now marketed by Pfizer in Australia, are efficacious under Australian conditions (Eggleton et al. 1991) but were less successful when used in a British study (Fontaine et al. 2006). Vaccination of goats with Glanvac resulted in significant protection against subsequent experimental inoculation with *C. pseudotuberculosis*, as evidenced by a decreased number of lesions (Brown et al. 1986). CLA vaccines are produced in the United States by Colorado Serum Company (Caseous D-T™ and Case-Bac; Piontkowski and Shivvers 1998). Fort Dodge Animal Health also produces a vaccine (Biodectin®). A live attenuated strain of *C. pseudotuberculosis* has been licensed for use as a vaccine in Brazil and is being developed further by the Empresa Baiana de Desenvolvimento Agrícola (www.ebda.ba.gov.br).

CLA remains prevalent in the field in spite of the availability of commercial vaccines for a long time. Several experimental vaccines have been investigated. Rationally, attenuated strains, from which *pld* was deleted (Hodgson et al. 1992), offered some protection, but efficacy was improved by inserting a genetically toxoided PLD to provide anti-PLD immunity (Tachedjian et al. 1995). Rationally, attenuated strain Toxminus has also been evaluated as a live vector for delivery of heterologous antigens (Moore et al. 1999, 2001). Live, attenuated vaccines have been produced against other pathogens (e.g., *Salmonella*) by deleting genes in the aromatic amino

acid biosynthetic pathway. This approach has also been tested in *C. pseudotuberculosis* with an *aroQ* mutant, but this mutant strain offered no useful protection (Simmons et al. 1998). A DNA vaccine expressing genetically attenuated PLD fused to cytotoxic T-lymphocyte antigen 4 to target antigen presenting cells yielded 70% efficacy in a challenge trial (Chaplin et al. 1999). A 40-kDa antigen, identified by isolation and screening of antibody-secreting cells from the immediate area of infected lymph nodes, was partially protective (Walker et al. 1994). Other studies identified immunodominant antigens by probing the proteome with immune sera from infected goats and sheep (Muckle et al. 1992; Braithwaite et al. 1993; Paule et al. 2004). These antigens may be useful in diagnostics or vaccines but have not yet been investigated in these contexts. Application of recent rapid advances in whole genome sequencing (http://rgmg.cpqrr.fiocruz.br) is likely to provide new opportunities to identify other virulence determinants and develop new strategies for design of vaccines.

OTHER *CORYNEBACTERIA*

A wide range of other *Corynebacterium* spp. have apparent pathogenic effects in animals (table 8.1), but most of these diseases are rare or mild. The limited importance of these diseases has restricted work to understand underlying virulence mechanisms.

Corynebacterium bovis primarily colonizes the bovine teat canal and is generally considered mildly pathogenic. It is nonetheless highly contagious, often being transmitted via contaminated milking equipment (Bramley et al. 1976). *C. bovis* causes occasional udder infections, with a mild increase in somatic cell count and a slight reduction in milk production. The organism is sometimes the only organism found in milk from cows with clinical mastitis, but the open question is whether it is the primary pathogen or an incidental finding due to its common asymptomatic carriage on the teat. Colonization with *C. bovis* may (Linde et al. 1980; Brooks and Barnum 1984) or may not (Honkanen-Buzalski et al. 1984; Hogan et al. 1988) protect against infection with the major mastitis pathogen *Staphylococcus aureus*.

Corynebacterium kutscheri is a common pathogen of laboratory rats, mice, and Syrian hamsters. Healthy rodents carry *C. kutscheri* as a commensal of the oral and nasal cavities and colorectum (Amao et al. 1995). Stressed animals are immunosuppressed, and a *C. kutscheri* infection (pseudotuberculosis) can develop as a result. A 100% infection rate can occur in an animal facility. Susceptibility varies with mouse strain, and male imprinting control region (ICR) mice males are more susceptible than females (Komukai et al. 1999). The organism is apparently present outside of laboratory facilities; the most recent and reliable diagnosis of human infection by the organism was in an infant bitten by a rat (Holmes and Korman 2007). It has also been isolated from wild voles (Barrow 1981).

C. kutsheri expresses a glycoprotein with nonspecific immunostimulatory effects on cytokine production by splenocytes and macrophages, and infections increase expression of host galectin-3, a powerful proinflammatory signal molecule (Kita et al. 1992; Won et al. 2007). These immunostimulatory properties may contribute to the nonspecific resistance induced by *C. kutsheri* but, when uncontrolled, may also lead to pathology.

Corynebacterium renale, *Corynebacterium pilosum*, and *Corynebacterium cystitidis* were all originally classified as serological types of *C. renale* but were subsequently recognized as separate species (Yanagawa and Honda 1978). They all cause bovine cystitis and pyelonephritis, but *C. renale* is most commonly isolated and is also found in cases of ovine cystitis and pyelonephritis and in caprine osteomyelitis (Higgins and Weaver 1981; Altmaier et al. 1994). Severe hemorrhagic cystitis with bladder ulceration, progressing to ureteritis and pyelonephritis, can be caused by *C. cystitidis* and is more severe than the generally mild cystitis caused by *C. pilosum*. These organisms are among the normal microflora in the bovine reproductive tract and can survive long periods in soil. Thus, there may be a cycle of infection, from contaminated urine to pasture to reinfection of the urinary tract (Hayashi et al. 1985). A distinguishing characteristic of *C. renale* is production, by most isolates, of an extracellular protein, renalin. This protein lyses erythrocytes in synergy with *S. aureus* β toxin and may be a virulence factor. The staphylococcal toxin apparently hydrolyzes sphingomyelin, producing ceramide, and then renalin interacts nonenzymatically with ceramide to lyse cells (Bernheimer and Avigad 1982). Experiments in rats have indicated that the potent urease of *C. renale* plays a crucial role in establishment of pyelonephritis (Jerusik et al. 1977). Adherence to host cells is also a vital step in pathogenesis, and pili of *C. renale* play a focal role;

antibodies to pili block adherence to cultured cells and to the mucous membrane of the mouse bladder (Honda and Yanagawa 1975, 1978).

C. ulcerans is the causative agent of several animal diseases, including bovine mastitis (Lipsky et al. 1982; Watts 1988) and other infections of camels, squirrels, dogs, cats, monkeys, killer whales, and lions (Tejedor et al. 2000; Seto et al. 2008). PLD, similar to that of *C. pseudotuberculosis*, is produced by all isolates of *C. ulcerans*; this toxin is presumed to be important in pathogenesis, but this has not been experimentally verified (McNamara et al. 1995). Some isolates of *C. ulcerans* are lysogenized by corynephage β, which carries *tox* and encodes diphtheria toxin (Schuhegger et al. 2008; Seto et al. 2008); in humans, these strains have been found in clinical cases presenting with diphtheria. Other potential virulence factors have been identified, including urease and iron-scavenging mechanisms (Riegel et al. 1995; Kunkle and Schmitt 2007).

The closely related *Corynebacterium urealyticum* and *Corynebacterium jeikeium* are primarily opportunistic pathogens of humans but may also cause urinary tract infections in animals. Knowledge of these organisms has taken a giant step forward with elucidation of the complete genomic sequence of a human clinical isolate of each species (Tauch et al. 2005, 2008). Genomic information has revealed several genes predicted to encode proteins important in the host/pathogen interaction and virulence; examples are surface-anchored attachment factors, secreted enzymes, and the urease of *C. urealyticum.* The newly developed next generation high-throughput DNA sequencing instruments usher in a new era in microbiology where it is quite feasible that complete genomic data will be available for every bacterium of interest. The background information provided by this technology changes the nature of the studies that can be carried out and is precipitating a revolution in the specific and comparative analysis of bacterial pathogenesis. The future challenge will be to devise efficient ways to determine, via this comprehensive genomic information, the function of genes and to screen for useful properties, such as vaccine efficacy.

ARCANOBACTERIUM

Members of the genus *Arcanobacterium* are small gram-positive, pleomorphic, but generally rod-shaped, bacteria that are nonmotile and non-spore forming. The genus was created by reclassification of *Corynebacterium haemolyticum* to *Arcanobacterium haemolyticum*, based on cell wall fatty acids and peptidoglycan components that differed from those of corynebacteria (Collins et al. 1982). The peptidoglycan of arcanobacteria contains lysine, rhamnose, and glucose, but not mycolic acid. Characterization of 16S rRNA sequences has been a useful tool in the classification of other bacteria to the genus *Arcanobacterium*, including *Actinomyces pyogenes* and *Actinomyces bernardiae* (Ramos et al. 1997), and the classification of the other species of *Arcanobacterium* has been principally directed by the sequencing of 16S rRNA genes (Lehnen et al. 2006). Eight species now comprise the genus *Arcanobacterium*, six of which were isolated from animals (table 8.2). *A. pyogenes* is the only species isolated from animals that has been studied in any detail.

ARCANOBACTERIUM PYOGENES

Arcanobacterium pyogenes is the most widely reported *Arcanobacterium* species It has been implicated as the cause of various diseases in a wide variety of animals, including all major domesticated food animals. It is often found among the resident microbiota on mucous membrane (gastrointestinal, genital, and upper respiratory) and skin of healthy animals. It is isolated from the bovine rumen (Narayanan et al. 1998) and the stomach of pigs (Jost et al. 2002a).

Arcanobacterium pyogenes is an opportunistic pathogen that causes suppurative infections in a variety of organs, including the skin, joints, testes, and visceral organs. Infections generally follow damage to mucous membranes by direct physical trauma, immunological action, or infection by other microorganisms. *A. pyogenes* infections have been found in a very wide variety of mammals and even some birds, but infections are rare in humans.

Pathogenesis

Arcanobacterium pyogenes causes suppurative infections, in the form of abscesses, empyemas, and pyogranulomas, often in concert with other bacteria, mainly non-spore-forming anaerobes recruited from resident commensal bacterial populations (e.g., *Corynebacterium*, *Bacillus*, *Bacteroides*, *Fusobacterium*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Staphylococcus*, and *Streptococcus*). Infection can result in abortion, arthritis, endocardi-

Table 8.2. *Arcanobacterium* spp. Infections in Animal Hosts

Arcanobacterium spp	Host	Disease[a]
bernardiae	Human	Abscesses, septicemia
bialowiezense	Bison	Edema and necrosis of skin
bonsai	Bison	Edema and necrosis of skin
haemolyticum	Human	Pharyngitis, wound infections
hippocoleae	Horse	Vaginitis
phocae	Seal	Superficial abscesses
	Otter	
	Dolphin	
pluranimalium	Deer	Lung abscess
	Porpoise	Unknown
pyogenes	Cattle	Mastitis, endocarditis, liver abscess, endometritis,
	Deer	abortion
	Goat	Pneumonia, intracranial abscesses
	Pig	Mastitis, abscesses
	Poultry	Pneumonia, endocarditis, septic arthritis
	Sheep	Osteomyelitis, nephritis, pneumonia, endometritis, abortion, orchitis

[a]An association with certain diseases has been noted, but, in most cases, there is no definitive proof that the *Arcanobacterium* isolate is the causative agent.

tis, mastitis, pneumonia, osteomyelitis, infertility, and vesiculitis. Abscesses are often heavily encapsulated, resulting in ineffectiveness of antibiotic therapy.

Liver abscesses, mastitis, and abortion are the most commonly reported diseases involving *A. pyogenes* infections in cattle. In liver abscesses, *Fusobacterium necrophorum* is the primary etiological agent with *A. pyogenes* being the second most frequently isolated pathogen. The disease is commonly found in cattle from grain-feeding programs with incidence up to 30% in some feedlots. Dietary and management factors influence the incidence. A sudden change to high-energy grain diets can lead to acidosis and ulceration of the rumen, which provides an entry point for pathogens to invade the blood stream and be deposited by portal circulation in the liver, with subsequent abscess formation (Nagaraja and Lechtenberg 2007). Economic consequences can be significant since cattle with liver abscesses have reduced feed intake, reduced weight gain, decreased feed efficiency, and decreased carcass dressing percentage (Brink et al. 1990).

Because *A. pyogenes* is commonly found in the normal commensal microflora of susceptible species, the incidence of disease is sporadic and depends on the presence of precipitating stresses or physical damage. *A. pyogenes* can cause severe mastitis in cows, with bacteria entering via injured teats from contaminated milking equipment. Clinical cases may be mixed infections. So-called summer mastitis may be spread by flies, which carry the organism to traumatized teats (Hillerton et al. 1990).

Arcanobacterium pyogenes is the most common opportunistic bacterium causing sporadic abortions. Such abortions may occur at any stage in gestation but are more common in the second half of gestation. *A. pyogenes*-induced abortion is sometimes associated with concomitant bovine viral diarrhea virus infection (Kirkbride 1993), but production of disease with pure cultures indicates that *A. pyogenes* can act as a primary pathogen (Semambo et al. 1991).

Orchitis in rams (Gouletsou et al. 2004) and mastitis in both dry and lactating cows (Hillerton and Bramley 1989) have been experimentally reproduced via inoculation with pure cultures of *A. pyogenes*. Such experimental infection systems, coupled with the ability to construct specific directed mutants in some isolates of *A. pyogenes* (Jost et al. 1999, 2002b), offer the opportunity to test engineered mutant strains for virulence in definitive hosts.

Virulence Factors

Actinomyces pyogenes produces several virulence factors, including tissue-damaging toxins and enzymes and attachment and colonization factors. It can cause a variety of diseases in multiple species, but there is no indication that particular virulence factors are associated with specific disease processes or particular hosts. Proteins suggested to be virulence factors (pyolysin [PLO], collagen binding protein, neuraminidase, and DNase) are ubiquitous among isolates (Lammler and Blobel 1988; Silva et al. 2008).

PLO is secreted by all strains of *A. pyogenes*. It is a pore-forming, cholesterol-dependent cytolysin (CDC) and is a major virulence determinant. *plo* mutants have reduced virulence in a mouse model (Jost et al. 1999). The properties and mode of action of PLO can, to some degree, be inferred from its similarity to other CDCs, which bind to cholesterol-containing rafts in the eukaryotic cell membrane, forming pores and resulting in death of the cell (Giddings et al. 2003; Rosado et al. 2008). They promote cytolysis of immune cells and alter host cytokine expression (Houldsworth et al. 1994; Nishibori et al. 1996; Ruiz et al. 1998). PLO is also responsible for the hemolysis observed when *A. pyogenes* is grown on blood-containing media. PLO is dermonecrotic and lethal when delivered IV or IP. The specific action of PLO *in vivo* is unknown, but it is assumed that its pleotrophic effects include damage to host cell membranes, with concomitant influences on the manifestation of disease. PLO expression is regulated *in vitro*, with increased expression during early stationary phase. Expression of PLO may be regulated by a two-component sensor kinase system (Jost and Billington 2005).

Extracellular matrix-binding proteins recognize components of the host tissue matrix to which *A. pyogenes* adheres as a necessary first step in colonization and persistence. *A. pyogenes* can bind to collagen, fibrinogen, and fibronectin; *cbpA*, the structural gene for collagen-binding protein, was found in 48% of North American strains (Esmay et al. 2003). Recombinant CbpA binds strongly to collagen types I, II, III, IV, and XI and weakly to types V and IX (Pietrocola et al. 2007) but does not bind to denatured collagen (Esmay et al. 2003). CbpA also binds to fibronectin (Pietrocola et al. 2007) via a subsite different from that involved in collagen binding. A *cbpA* mutant had a greatly reduced capacity to adhere to epithelial and fibroblast cell lines (Esmay et al. 2003), and antibodies to CbpA blocked the interaction of CbpA with collagen (Pietrocola et al. 2007). The importance of collagen binding in colonization is not clear, as collagen is not exposed in normal healthy tissue; therefore, CbpA may be principally involved with adherence to damaged tissue, or other properties may be more important.

Other potential adherence factors, such as fimbriae and a 20-kDa fibronectin-binding protein, have been reported (Jost and Billington 2005), but no detailed characterization is yet available.

Actinomyces pyogenes produces neuraminidase, and two neuraminidase genes have been identified. *nanH* has been found in all isolates, whereas *nanP* was in 64% of isolates in one study (Jost et al. 2002b) and 100% of isolates in a second study (Silva et al. 2008). Both proteins are cell wall anchored. Neuraminidases cleave terminal sialic acid residues from carbohydrates and glycoproteins. In some bacteria, this exposes cryptic host cell receptors and allows bacterial adhesion. In *A. pyogenes*, a double mutant with no neuraminidase activity had reduced ability to bind to HeLa cells, indicating an important role for neuraminidases in adhesion (Jost et al. 2002b).

Actinomyces pyogenes expresses other enzymes that may play some role in virulence. Zymogram-based studies have found five protein bands on SDS-PAGE with protease activity; all degraded gelatin, but three lower molecular weight proteins degraded only casein. They were tentatively identified as Ca^{2+}-dependent serine proteases (Takeuchi et al. 1995). No mutant studies have been carried out to investigate a definitive role for proteases in *A. pyogenes* pathogenesis. However, antibodies to proteases were much more prevalent in pigs with *A. pyogenes* abscesses (93%) than in control (35%), suggesting that proteases are expressed during the disease process (Takeuchi et al. 1979).

All *A. pyogenes* isolates produce a secreted DNase (Lammler and Blobel 1988). DNase may aid in the release of bacteria from disintegrating host cells in inflammatory lesions by digesting host DNA and reducing local viscosity. The action of DNase may also make nucleotides available for bacterial utilization.

Vaccines

Vaccination with crude whole cells or culture supernatant of *A. pyogenes* has not offered any useful level of protection to sheep or cattle (Ding et al.

1998; Hunter et al. 1990), but some success has been seen in a mouse challenge model (Cameron et al. 1976). Vaccination with formalin-inactivated PLO, recombinant PLO, and genetically attenuated PLO gives protective immune responses in mice (Jost et al. 1999, 2003), but similar studies have not been conducted in host animals. Healthy animals naturally colonized with *A. pyogenes* often have antibodies to the organism and to PLO, but these offer no protection from disease. Vaccination with whole cell and culture supernatant can increase antibody titers without giving any protection. This has led to doubts that serum antibodies offer protection, even though passive immunization of mice with PLO-specific serum prevented *A. pyogenes* infection (Billington et al. 1997). To date, no other potential antigens have been tested in challenge trials.

FUTURE DIRECTIONS

Elucidation of the factors involved in virulence of corynebacteria and arcanobacteria (particularly *A. pyogenes* and the high-profile *Corynebacterium* spp.) is justified by their impact on animal production worldwide. Interest in *C. pseudotuberculosis* and CLA is highest in areas of the world where small ruminants play a major role in the agricultural economy. Clear understanding of virulence of this organism, leading to effective, easily delivered means of prevention, will have major impact on many nations but especially on the developing world. *A. pyogenes* is isolated virtually every day from multiple species in veterinary diagnostic laboratories around the world and is thought by some to be the most widely distributed, most common opportunistic pathogen of mucosal surfaces in domestic animals. The economic impact of these infections is much more difficult to estimate than those resulting from the dramatic, epidemic disease affecting animal production, but they must be substantial. There are many opportunities to have major impact on these losses, beginning perhaps with a more fundamental understanding of the biology of pathogenic corynebacteria and arcanobacteria through application of high-throughput genomics. Rational evaluation of the resulting data and *in vivo* testing of virulence hypotheses should yield targets for therapeutic and vaccination approaches.

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9
Rhodococcus

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INTRODUCTION

The genus *Rhodococcus* currently (2009) contains about 43 species (www.bacterio.cict.fr/qr/rhodococcus.html), several of which are of interest because of their diverse metabolic activities; their industrial applications as producers of bioactive steroids, acrylamide, and acrylic acid; or their potential in bioremediation and fossil fuel biodesulfurization. Two species are significant as pathogens, *Rhodococcus equi* in mammals and *Rhodococcus fascians* in plants. This chapter focuses entirely on *R. equi*, with emphasis on recent findings. References supporting some of the uncited but generally accepted statements in this chapter may be found in previous editions of this book.

Rhodococcus equi is an important cause of pneumonia in foals, usually between 4 and 12 weeks of age. Infection causes a subacute or chronic abscessating bronchopneumonia, sometimes with ulcerative typhlocolitis, and may include mesenteric lymphadenitis, osteomyelitis, purulent arthritis, reactive arthritis, and ulcerative lymphangitis. Infection in adult horses is rare. Tuberculosis-like lesions caused by *R. equi* may also occur in the submandibular and other lymph nodes of cattle and pigs. In young goats, granulomatous lesions in the liver are associated with wasting and death. Infection in other animal species is rare and usually the result of immunosuppression. *R. equi* is an important cause of AIDS-associated pneumonia in HIV-infected or otherwise seriously immunocompromised humans. The organism has a worldwide distribution, but infection in foals is most common in regions with long, hot summers.

CHARACTERISTICS AND SOURCES OF THE ORGANISM

Rhodococcus equi is an actinomycete member of mycolata, a suprageneric taxon that includes *Corynebacterium*, *Mycobacterium*, and *Nocardia* and shares the characteristic lipid-rich, branched-chain, mycolic-acid-dominated cell envelope of these GC-rich organisms. It is a gram-positive, obligately aerobic coccobacillus that grows well on nonselective media after 48 h of culture at 37°C. *R. equi* is a soil organism, certain specific virulent types of which are associated particularly with horses, cattle, or pigs. Adaptation of different types to these particular hosts (horses, cattle, pigs) is mediated by the presence of host-associated plasmids (discussed below).

On some horse farms the clinical disease is endemic and devastating, whereas it is sporadic on others and not recognized on most. This difference reflects variation in numbers and virulence of isolates as well as in environmental conditions (temperature, dust, soil pH), management, and other factors (Muscatello et al. 2007). Since volatile fatty acids in manure enhance its growth, the bacterium occurs in largest numbers where horses (or other herbivores) are present. Importantly, *R. equi* can also replicate in the intestine of foals up to about 3 months of age, enhancing the dissemination of virulent bacteria swallowed by pneumonic foals (Takai 1997). Over time, there may be a progressive increase in infection to untenable levels. Inhalation of contaminated dust particles or the infected breath of sick foals leads to pneumonic infection in foals (Muscatello et al. 2006, 2009). Endemic farms are

generally those used intensively to rear foals over many years, where summer temperatures are high, soil type is sandy, and dust is extensive. Farms with endemic disease are more heavily infected with virulent *R. equi* than those where the disease is absent or sporadic.

BACTERIAL VIRULENCE FACTORS

Genome Analysis

The genome of *R. equi* 103, a foal pneumonic isolate that is readily transformable, has been completely sequenced and annotated (Letek et al. 2008a). It is 5.04 Mb and encodes 4598 predicted genes including 73 on the virulence plasmid. Genome comparisons show that the *R. equi* genome is most similar to, but far smaller than, that of *Rhodococcus jostii* (RHA1), the only other *Rhodococcus* for which the genome has been completely sequenced. Interestingly, only 13 pseudogenes were identified in the *R. equi* genome, indicating a very low rate of gene decay. Among the striking features of the genome are the large number of surface-associated proteins (1057 genes), many (406) of which are transporters, the large secretome (610 genes), and the many regulatory genes (406 genes), consistent with the variety of niches colonized by an organism with both a saprophytic and pathogenic lifestyle. There is a large set of genes involved in lipid catabolism but no genes involved in sugar transport, emphasizing the importance of lipids as the major carbon source for the organism. Some members of the mycobacterial PE/PPE (proline glutamic acid/proline proline glutamic acid) multigene family are present. Part of the importance of *R. equi* is that it is a pathogen of macrophages related to *Mycobacterium tuberculosis*. Once details of its pathogenesis are fully understood, the comparative analysis of this knowledge may enhance understanding of the key events that allow mycobacteria and related bacteria to persist and replicate in macrophages. Nevertheless, its genome has many differences from that of *M. tuberculosis*. For example, it possesses 23 two-component system sensor kinases and response regulators, more than twice the number possessed by the host-adapted, non-free-living *M. tuberculosis*. The availability of the completed genome of *R. equi* (www.sanger.ac.uk/Projects/R_equi/) is a major advance in the field. When combined with the recent advances in the development of a reliable counter-selectable mutation system to produce unmarked gene deletions (Van der Geize et al. 2008) and ability to produce stable site-specific integration (Hong and Hondalus 2008), there is potential for considerable advances in understanding the pathogenesis of *R. equi* infection.

Virulence Plasmids

Foal-Virulent R. equi

A major advance in understanding the virulence of *R. equi* was the discovery by Takai et al. (1991a) that virulence in mice is related to the production of characteristic proteins described by them as 15- and 17-kDa proteins. A second major advance was the recognition that all isolates that produce these proteins contain a large related plasmid ranging in size from 80 to 90 kb. All isolates from foals with the clinical disease possess these plasmids and express these proteins (Takai et al. 1991b; Tkachuk-Saad and Prescott 1991). The virulence-associated proteins recognized initially are actually produced by one gene, designated *vapA* by Tan et al. (1995). VapA is a surface-expressed protein, apparently anchored at its N-terminal end to the cell envelope by an unusual lipid modification, which partially accounts for the variable number of the protein bands on SDS-PAGE (Tan et al. 1995). Its expression is thermoregulated, occurring between 34 and 41°C (Takai et al. 1992). Expression is enhanced by low pH (Takai et al. 1996a). Virulent strains cured of the large plasmid show a dramatic decrease in lethality in mice and become avirulent for foals. In contrast to the parent, plasmid-cured strains also fail to survive and to replicate in macrophages. Jain et al. (2003) used gene deletion and complementation to provide convincing proof of the central role of VapA in virulence, in contrast to VapsC–F.

Two virulence plasmids from foal isolates have been sequenced (Takai et al. 2000; Letek et al. 2008b). These are identical and contain 73 open reading frames (ORFs), of which 26 are arranged in a discrete region of 21.3 kb, which fulfills the criteria for a typical pathogenicity island (PAI). This pathogenicity region contains a total of nine members of the *vap* multigene family, of which six encode full-length Vap proteins (*vapA*, *C*, *D*, *E*, *G*, and *H*) and three are degenerate *vap* genes or pseudogenes (*vapF*, *I*, and *X* ; Letek et al. 2008b). In contrast to VapA, VapsC–E are secreted from the cell into the environment (Byrne et al. 2001). The function of the Vap proteins is unknown. Apart from the

vap family genes, 9 of 26 ORFs in the pathogenicity island (PI) lacked similarity to genes of known function (Letek et al. 2008b). Two genes with similarity to regulatory genes are present on the PI, a LysR-family transcriptional regulator and a two-component response regulator. Subsequent sequencing of, and comparison with, a *vapB*-positive virulence plasmid, typically associated with pig isolates, showed that the PI carried in this type of plasmid was smaller (15.5 kb) than that of the foal isolate and differed extensively in the region associated with *vap* genes, although 6.2 kb was conserved between the two PAI regions (fig. 9.1). In particular, five ORFs associated with the *virR* operon (discussed below) were conserved (ORF4–ORF8, comprising the LysR-regulatory gene *virR* and ORF8, an orphan two-component regulatory gene, with a *vap* gene and a putative major facilitator superfamily transporter [*orf5*] contained within the operon). The conserved non-*vap* gene PI elements include *lsr2*, encoding a DNA-bridging histone-like protein also found in *Mycobacterium*, *orf21* (*scm2*), encoding a protein similar to AroQ* class-secreted chorismate mutase, and *orf23*, encoding an s-adenosylmethionine-dependent methyltransferase. The conservation of these genes between the two virulence plasmid PAI regions suggests their importance in pathogenesis. In *M. tuberculosis*, the Lsr2 protein protects the organism against reactive oxygen intermediates during macrophage infection (Colangeli et al. 2009). Apart from the PI, the remainder of the large virulence plasmid of *R. equi* contains the "housekeeping" regions (conjugation region, partition region, and a region of unknown function) with extensive homology to those found in the large plasmid of the environmental organism *Rhodococcus erythropolis* (Letek et al. 2008b). The replication region of the basic plasmid backbone seems to contain a "hot spot" for the insertion of foreign DNA carried by mobile genetic elements, which seems to be critical for the niche-adaptive evolution of these circular rhodococcal replicons (Letek et al. 2008b).

Pig Virulent R. equi

Isolates from the submaxillary lymph nodes of infected pigs also contain a large virulence plasmid. This lacks *vapA* but has a closely related gene, *vapB,* which encodes a 20-kDa protein (VapB) that confers an "intermediate" virulence phenotype for mice (Takai et al. 1996b; Ocampo-Sosa et al. 2007). The sequence analysis of *vap* genes on the *vapB* plasmid PAI (Letek et al. 2008b) has provided important insights into the evolution of *vap* genes and their likely role in adaptation of *R. equi* to different mammalian hosts. Five new members of the *vap* multigene family (v*apJ*, *vapK1*, *VapK2*, *vapM*, and *vapL*) encode products with the conserved C-terminal domain likely to contain the active site of the protein, as well as more divergent sequences. A model of the deduced evolutionary dynamics of the *vap* gene family suggests derivation by gene duplication followed by sequence diversification, rearrangement through homologous recombination, and gene decay from a common plasmid ancestor originally containing four *vap* genes (Letek et al. 2008b).

Other R. equi

Opportunistic infections in immunocompromised human patients can be caused both by mouse highly and intermediately virulent and by mouse avirulent *R. equi* isolates, so that the pathogenesis of *R. equi* infection in these patients is different from the pathogenesis in foals and pigs, whose isolates always possess the virulence plasmid (Takai 1997). In some cases, however, AIDS patients are infected with *R. equi* that appear to have either a horse or a pig source. Isolates from goats (Tkachuk-Saad et al. 1998) lack a virulence plasmid. However, cattle isolates, associated with tuberculosis-like lesions in cervical lymph nodes, are commonly parasitized by traA+/vapAB– plasmids, although a small proportion of them carry VapB-positive plasmids (Ocampo-Sosa et al. 2007). These plasmids thus appear to have a backbone similar to those of the vapA and vapB plasmids, as indicated by the presence of the *traA* gene, yet lack the *vapA* and *vapB* genes. Further work is required to characterize these plasmids. Since about one quarter of human isolates carry plasmids with the traA+/vapAB– signature (Ocampo-Sosa et al. 2007), it is possible that cattle may be a source of infection for some immunocompromised humans.

Virulence of non-plasmid-containing human isolates of *R. equi* has also been associated with β-lactam antibiotic resistance. Compared with susceptible isolates, β-lactam-resistant isolates have cell surface "appendages" resembling to some extent the tail of bacteriophages, and bacteriophage-like particles were found only in the culture supernatant of appendage-producing isolates (Nordmann et al. 1994).

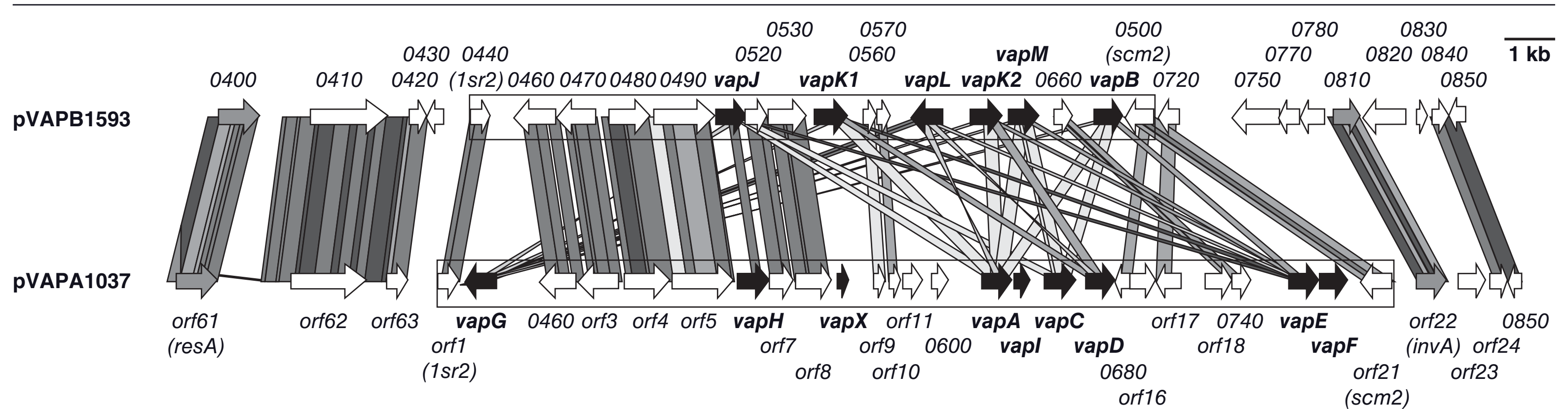

Figure 9.1. Genetic structures of the variable region of the *R. equi* virulence plasmids pVAPB1593 (pig type) and pVAPA1037 (horse type) and sequence comparision using the Artemis comparison tool (www.sanger.ac.uk/Software/Artemis/). Regions with significant similarity (tBlastx) are connected by colored lines (red, sequences in direct orientation; blue, sequences in reverse orientation). The intensity of the color indicates the strength of the sequence homology (pink/light blue, lowest; red/deep blue, highest). The virulence-associated *vap* genes are shown in black, and the mobility-related *resA*- and *invA*-like genes are shown in gray. The DNA region corresponding to the *vap* PAIs (boxed) were defined using the "Alien Hunter" algorithm (www.sanger.ac.uk/Software/analysis/alien_hunter/), which identifies horizontally acquired DNA by reliably capturing local compositional biases. Gene designations for pVAPB1593 according to standardized annotation nomenclature adopted for *R. equi* virulence plasmids (pVAP) (Letek et al. 2008b), those for pVAPA1037 according to the nomenclature used by Takai et al. 2000 (except for newly identified genes during the reannotation by Letek et al. 2008a). From Letek et al. (2008b), with permission, American Society for Microbiology. (See color plate)

Other Virulence Determinants

The cell envelope of *R. equi* is probably an important virulence feature of the organism. A unique feature of the mycobacterial cell wall is the presence of a lipid permeability barrier or layer external to both the plasma membrane and the peptidoglycan–arabinogalactan cell wall matrix (Sutcliffe 1998). This lipid permeability barrier or envelope is composed of mycolic acids (3-hydroxy-2-alkyl branched fatty acids) bound to the arabinogalactan wall polysaccharide and to other cell envelope lipids such as trehalose mycolates. Sutcliffe (1998) has suggested that *R. equi* possesses a similarly composed cell envelope, with a range of glycolipids present in the outer leaflet of the lipid bilayer. The presence of an asymmetric outer lipid permeability barrier predicates that the cell envelopes of the mycolata contain permeability pathways for hydrophilic solutes. Two such channel-forming proteins have recently been described in *R. equi* (Riess et al. 2003).

In *M. tuberculosis*, mycolic acids may be factors important for survival of mycobacteria inside the phagocyte by protecting the cell wall and membrane infrastructure from the effects of lytic enzymes and by scavenging potentially harmful reactive oxygen intermediates. They may have a similar role in *R. equi*. In *M. tuberculosis*, they are considered an important target for antimycobacterial drug development (Bhatt et al. 2007). Cell wall mycolic acid-containing glycolipids may contribute to virulence of *R. equi*. As determined by lethality and granuloma formation in mice, strains with a longer carbon chain mycolic acid were more virulent than those with shorter chains (Gotoh et al. 1991). More recently, trehalose 6,6'-dimycolate, a compound identified in *R. equi*, was shown to be the main granulomatogenic mycolyl glycolipid isolated from a *Rhodococcus* sp. (Ueda et al. 2001). A transposome mutation of the mycolyl transferase gene *fbpA*, a homologue of the *M. tuberculosis* Antigen 85 *fbpA* gene, was found to have impaired capsule synthesis (Sydor et al. 2008). Although the mutant was unaffected in mouse clearance and macrophage cytotoxicity, it was more cytotoxic than wild-type and even multiplied to some extent in macrophages, suggesting that capsule is not an important virulence factor in *R. equi* and may even counteract virulence traits. Interestingly, mycolyl transferases with high homology to the Antigen 85 complex were identified by proteomic analysis of secreted proteins of *R. equi* (Barbey et al. 2008).

A lipoglycan, lipoarabinomannam (LAM), may localize to both the plasma membrane and the outer lipid layer (Sutcliffe 1998) and play a role in virulence. The LAM of *R. equi* (ReqLAM) was shown to be smaller than and distinct from mycobacterial LAM, and to lack the extensive arabinan branching of mycobacterial LAM but to retain side chains containing terminal mannose units. These latter mannose units are particularly important in interaction of the *M. tuberculosis* with macrophages. In *M. tuberculosis*, mannose-capped LAM (ManLAM) is involved in inhibition of phagosome maturation and of apoptosis, as well as inhibition of macrophage release of interferon-γ and of IL-12 release by dendritic cells, thus contributing to modulation of the immune responses (Briken et al. 2004). The inhibitory activity of *M. tuberculosis* ManLAM, which in mice macrophages appears to be mediated by Toll-like receptor 2, is in striking contrast to the pro-inflammatory effects of phospho-*myo*-inositol capped LAM isolated from nonpathogenic mycobacteria (Briken et al. 2004). Incorporation of *M. tuberculosis* ManLAM into macrophage lipid rafts is a prerequisite for the phagosomal maturation block characteristic of the pathogenesis of this infection (Welin et al. 2008). There is no suggestion, however, of a down-regulatory immunomodulatory effect of *R. equi* ManLAM since purified ReqLAM induced inflammatory and regulatory cytokine mRNA expression from equine macrophages cultured *in vitro* in a manner similar to those induced by live virulent *R. equi* (Garton et al. 2002).

The cholesterol oxidase, ChoE, of *R. equi* is a prominent exoenzyme that has been suspected to be important in cellular membrane destruction and in providing nutrient sources for the organism within the macrophage, as well as causing tissue damage. In *M. tuberculosis*, the cholesterol oxidase, ChoD, is required for virulence (Brzostek et al. 2007). Unexpectedly, however, mutation of *choE* in *R. equi* did not result in loss of virulence (Pei et al. 2006), perhaps because the organism could compensate for loss of ChoE through other cholesterol oxidases and membranolytic enzymes, such as ChoD, the gene that is found in the genome.

Many aspects of virulence in *R. equi* are currently unknown or speculative, but details are slowly becoming clarified. For example, mutation of high-temperature requirement A (*htrA*, a chaperonin) and nitrate reductase (*narG*) resulted in complete attenuation in a mouse clearance model and could

be complemented in the case of *htrA*. Mutation of peptidase D (*pepD*) resulted in partial attenuation, which could also be complemented (Pei et al. 2007a). These genes may therefore be involved in full expression of virulence in *R. equi*, but their role in survival of the organism in macrophages is highly speculative in the absence of other information. Teasing out the role of virulence, virulence-associated, and "housekeeping" genes (Wassenaar and Gaastra 2001) in the pathogenesis of *R. equi* infection will be complex and expensive but may be aided by the development of genome-wide microarrays and of mutants of two-component regulatory and other genes and examination of gene expression by mutants growing within macrophages (Rahman et al. 2005).

PATHOGENESIS

Overview

The basis of the pathogenicity of *R. equi* is its ability to multiply in and eventually to destroy alveolar macrophages, but the mechanisms are not understood. The infectivity of *R. equi in vitro* is largely or exclusively limited to cells of the monocyte-macrophage lineage. In contrast to macrophages, neutrophils from foals and adult horses are fully bactericidal, and such killing is markedly enhanced by a specific opsonizing antibody (reviewed by Hondalus 1997). The following discussion describes the basic processes of phagocytosis and killing by macrophages in the context of what is known about *R. equi*.

Interactions between the Bacterium and Host Defenses

Phagocytosis: Receptor Recognition and Internalization

The signals for and the mechanism of bacterial ingestion vary with the phagocyte receptor involved, which in turn also affects activation of the cell. Complement receptor (CR)-mediated phagocytosis of bacteria involves several macrophage receptors (CR1, CR3, CR4) that bind one or more of C3b, C3bi, and C4b complement components deposited on the bacterial surface. Whereas in antibody (FcR)-mediated phagocytosis, pseudopodia tightly engulf opsonized bacteria before drawing them into the macrophage, in CR-mediated phagocytosis, opsonized bacteria appear to sink passively into the macrophage without marked pseudopodia production and with a more irregularly attached phagosome membrane (Aderem and Underhill 1999). Several factors mediate these physical differences (Tjelle et al. 2000). FcR-induced phagocytosis is strongly associated with production of reactive oxygen intermediates (oxidative burst) and pro-inflammatory mediators, whereas CR-mediated phagocytosis does not induce this effect (Aderem and Underhill 1999). The effect of the phagocyte receptor involved in mediating differences in internalization mechanisms is illustrated also by the mannose receptor (MR), which recognizes mannose and fucose on bacterial surfaces. Unlike FcR- and CR-mediated phagocytosis, MR-mediated phagocytosis is not associated with accumulation of the cytoskeletal proteins vinculin and paxillin around the phagosomes (Aderem and Underhill 1999). Optimal binding of *R. equi* to macrophages *in vitro* requires complement and is mediated by Mac-1, a leukocyte CR type 3 (CR3; Hondalus and Mosser 1994). The ReqLAM binds to recombinant mannose-binding protein, which may activate complement C3b deposition onto *R. equi* via the lectin pathway, and thus also promote Mac-1-mediated uptake into macrophages (Garton et al. 2002). ReqLAM may be speculated also to bind other collectins since *M. tuberculosis* LAM is a ligand for human pulmonary surfactant protein A, and equine surfactant proteins bind mannose (Garton et al. 2002). In addition, entry to macrophages through ReqLAM binding to the MR may occur. Since opsonization of *R. equi* with a specific antibody is followed by increased phagosome–lysosome fusion and by significantly enhanced *R. equi* killing by equine macrophages, macrophage entry through non-Fc receptors may be important in determining the fate of these bacteria. The general limitation of the disease in foals to the period between 4 and 12 weeks after birth coincides with the time of major decline of maternally derived antibodies, supporting the concept that entry of *R. equi* into macrophages by non-Fc receptors may be important in allowing it to avoid antibody-associated macrophage killing pathways.

Phagocytosis: Maturation of the Phagosome

F-actin is removed from the phagosome once bacteria are internalized, and the phagosome undergoes a series of fusion and fission events with vesicles from the endocytic pathway, a complex maturation process that leads to the formation of phagolysosomes. The usual progressive movement of the

phagosome on microtubules into the cell during maturation allows the phagosome to interact with the endosomal system. During maturation, the phagosome membrane increasingly changes to resemble that of late endosomes and lysosomes, following a regulated and sequential pattern in which phagosomes change from ready fusion with both early and late endosomes to fusion most readily with lysosomes and finally to failure to fuse with any endocytic organelle. This maturation process is characterized by changes in the molecules associated with the phagosomal membrane from those typical of early endosomes (e.g., Rab5, annexin, N-ethylmaleimide-sensitive factor [NSF], transferrin receptor, MR) to those typical of late endosomes and lysosomes (e.g., Rab5, Rab7, mannose-6-phosphate receptor, cathepsin D, LAMP1 [lysosome-associated membrane protein1], LAMP2).

Maturation of the phagosome is also characterized by progressive acidification with delivery of the vacuolar proton pump from the early and late endosomes and removal of the Na^+/K^+-ATPase from the late endosome, so that the vacuolar pump works unopposed in the phagolysosome (pH ≤5.5). For bacteria that do not interfere with the process, phagocytosis to phagolysosome formation takes about 5 min.

How *R. equi* Behaves in Macrophages

Plasmid-encoded products contribute to the ability of *R. equi* to survive and replicate in macrophages. Possession of the virulence plasmid by foal isolates gives them the property of arresting phagosome maturation at a stage between the early and late endosomes (Fernandez-Mora et al. 2005; Toyooka et al. 2005). Although the mature *R. equi*-containing vacuoles possess late endosome markers, such as LAMPs and Rab7, they lack cathepsin D and proton-pumping ATPase and the ability to fuse with lysosomes (Fernandez-Mora et al. 2005). Since acidification does not occur, the *R. equi*-containing vacuoles have a pH of about 6.5. A soluble component of the supernatant of virulent *R. equi* is responsible for reduction of acidification (Toyooka et al. 2005) but has not been identified. In addition, cytotoxicity of *R. equi* for J774E murine macrophages is strongly up-regulated by the possession of the virulence plasmid (Lührmann et al. 2004), with macrophage death resulting from necrosis rather than apoptosis. The presence of transferrin in early endosomes may explain the importance of iron restriction in regulating transcription of some virulence plasmid PI genes (discussed below). Natural-resistance-associated macrophage protein 1 (NRAMP1) confers innate resistance to macrophages against the growth of certain intracellular pathogens (Russell 2001). A divalent cation transporter that shows preference for iron or manganese and pumps iron into the phagosome (Cellier et al. 2007), NRAMP1 is associated with efficient acidification of the phagocytic vacuole, although details of its mechanism of action are unclear (Russell 2001). Once inside the phagosome, iron stimulates formation of biotoxic reactive oxidative species via the Fenton/Haber–Weiss reactions. The limited quantities of iron transported into the phagosome by NRAMP1 to catalyze the generation of antimicrobial oxidative radicals do not increase iron availability for bacteria since iron in the phagosome is removed during the process of nitric oxide (NO) formation. Interestingly, variation in NRAMP1 genes has been significantly associated with development of *R. equi* pneumonia in foals (Halbert et al. 2006).

The specific lack of the vacuolar proton pump appears to be responsible for the mildly acid conditions. Inhibition of acidification blocks vesicular delivery to lysosomes, and the pH of the phagosome is probably important in controlling fusion events. pH in the phagosomes containing mycobacteria is maintained mildly acidic, possibly by persistence of the Na^+/K^+-ATPase or by ammonia production within the phagosome. Ammonia, which can be produced in several ways by *M. tuberculosis*, may contribute to inhibition of phagolysosome formation but whether the prominent urease of *R. equi* has a similar role is unknown.

Oxygen-Dependent Killing Mechanisms

Besides lysosomal degradation, macrophages can inhibit or kill bacteria by producing toxic reactive oxygen and nitrogen species through the respiratory burst phagocyte oxidase and inducible NO synthase (iNOS). Macrophage production of toxic superoxides from the relatively inert oxygen molecule involves production by a membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that is activated in the "respiratory burst," which occurs when opsonized bacteria initiate the phagocytosis process. NO synthetases, especially iNOS, catalyze the oxidation of a guanidino nitrogen of L-arginine to NO. iNOS is usually transcribed in response to cytokines (IFN-γ, IL-1,

TNF-γ) released in response to microbial products or through immune cell interactions. Both IFN-γ and TNF-α are required for clearance of virulent *R. equi* from mice (Kasuga-Aoki et al. 1999). Besides sometimes synergizing with the oxidative burst to produce peroxynitrite, NO and its derivatives have a longer lasting bacteriostatic effect on bacteria in the phagosome. For *M. tuberculosis* and probably for *R. equi*, once macrophages are activated by IFN-γ, these cells overcome the block that the bacterium imposes on endosomal maturation.

Killing of *R. equi* by murine macrophages was shown to be dependent on IFN-γ, which activated macrophages to produce reactive nitrogen and oxygen intermediates, which in turn combined to peroxynitrite ($ONOO^-$), the essential bactericidal factor (Darrah et al. 2000). IFN-γ activation of macrophages prevented growth of *R. equi*, in contrast to their replication in non-IFN-γ activated macrophages, consistent with the role of IFN-γ in activating the high-output iNOS (Darrah et al. 2000). The study suggests a two-step model for efficient killing of *R. equi* (Darrah et al. 2000). The first step, macrophage activation by IFN-γ and TNF-γ, results in NO production following iNOS transcription, and the second step, signalled by bacterial phagocytosis, results in the respiratory burst and the production of, and killing by, $ONOO^-$ produced by reaction between nitrogen and oxygen intermediates.

Influence of Macrophage Environmental Factors

The environment of the macrophage has a considerable effect on expression of PI genes associated with replication of the organism. Virulence in *R. equi*, which in foal isolates is focused on the PI and on VapA, appears to be both highly and complexly regulated. Considerable work is required to understand the signals and factors controlling virulence and the metabolic behavior of *R. equi* within the macrophage, but initial evidence has shown that expression of virulence genes is a function of temperature and pH, and of iron, magnesium, and hydrogen peroxide concentrations. This section discusses the macrophage environment as it affects the expression of virulence and metabolic behavior of *R. equi*; the following section contains a more detailed discussion of the transcriptional regulation of virulence.

Enhanced transcription of *vapA* and *vapD* under acid conditions was noted by Benoit et al. (2001), who also described a state of acid tolerance or adaptation by both virulent and plasmid-cured *R. equi*. Studies by Ren and Prescott (2003) using a microarray of the virulence plasmid genes showed that only genes on the PI are differentially transcribed inside equine macrophages in comparison to the isolate grown at 30°C *in vitro* under nonnutrient limiting conditions. Six *vap* genes were transcribed, with two other genes, *orf9* and *orf10*, being the most transcribed. *Orf8*, the two-component response regulator homologue, was mildly up-regulated. The major influence of PI transcription was temperature, but temperature-up-regulated genes were found to fall into two groups. Group I, which contained 12 PI genes including the six *vap* genes, were further up-regulated by low iron and down-regulated by low magnesium, whereas group II genes, *orf3*, *orf9*, and *orf10*, were up-regulated by low magnesium and down-regulated by low iron. VapA was the gene most responsive to regulation by environmental influences, with low iron and low pH independently markedly enhancing its transcription. The regulation of VapA expression by iron was demonstrated by Jordan et al. (2003), who also showed that virulence plasmid-positive *R. equi* was able to obtain iron from bovine transferrin and lactoferrin. It may be relevant that there is an association between *R. equi* mortality and transferrin genetic type (Mousel et al. 2003). In addition to these compounds, *R. equi* is also able to derive iron from heme and hemoglobin, which are abundant sources of iron in the host environment (Miranda Casoluengo et al. 2005). Since the concentration of free iron is extremely low in animal and human hosts, iron is used by many pathogens as an important signal controlling virulence gene expression. *R. equi*, like *Mycobacterium* species, contains two transcriptional regulators that respond to the iron concentration: IdeR (also known as DtxR) and FurA. Although both bind to DNA (and thus act as a transcriptional repressor) in the presence of iron, they are evolutionarily not related. As each recognizes an unrelated DNA sequence, they control the expression of different genes. In *Mycobacterium* species, FurA is associated with the oxidative stress response by controlling the expression of, among other proteins, catalase (Pym et al. 2001), whereas IdeR is controlling the transcription of genes involved in iron acquisition and storage (Gold et al. 2001). Interestingly, constitutive expression of IdeR in *M. tuberculosis* led to complete attenuation, demonstrating that IdeR plays a key role in virulence (Manabe et al. 1999). It seems

likely that FurA and IdeR play similar roles in *R. equi*, although this remains to be established.

An IdeR binding site was found at the −35 promoter region of the *vapA* gene, suggesting not only that the low iron environment of the macrophage is an important regulatory signal for production of VapA but that there is important regulatory interaction between the chromosome and the plasmid. A functional *ideR* gene had earlier been identified in *R. equi* by Boland and Meijer (2000). In contrast to previous reports, it was recently shown that *R. equi* produces at least one type of sidephore, which is a group of structurally diverse compounds with high affinity for iron. This catecholate siderophore is produced under iron-limiting growth conditions. Using a combination of random and site directed mutagenesis, the genes required for production of the siderophore and the subsequent uptake of the siderophore–iron complex were identified. As expected, the transcription of these genes is controlled by the iron concentration, with a 100-fold up-regulation of transcription of these genes under iron-limiting growth conditions. Surprisingly, mutants that were unable to produce or take up the catecholate siderophore were not attenuated in the mouse model. This shows that *R. equi* employs at least one additional high-affinity iron uptake system that is required for virulence (Miranda Casoluengo et al. 2005, 2008). The use of multiple iron uptake systems or the use of different systems for saprophytic and pathogenic lifestyles of pathogens is fairly common and appears to be a specific adaptation of the pathogen to a specific host environment.

Recognition that iron restriction regulates VapA expression has led to an important series of studies demonstrating the inhibitory effect of gallium maltolate on *R. equi* not only *in vitro* but also *in vivo* (Harrington et al. 2006; Martens et al. 2007a). Because gallium is a trivalent semimetal that is chemically similar to ferric ion, it can interfere with the growth of some intracellular bacteria, including *R. equi*, by interfering with iron metabolism. Should oral administration of gallium maltolate to young foals be shown to be effective in preventing or treating *R. equi* pneumonia, as seems feasible (Martens et al. 2007b), this would be an important example of development of nonantibiotic antimicrobial therapy based on understanding of the pathogenesis of an infection. There is obvious potential application to therapy of multidrug resistant *M. tuberculosis* in humans.

Rhodococcus equi is highly resistant to hydrogen peroxide, an oxidative stress that reflects the production of reactive oxygen intermediates effect during the oxidative burst of phagocytes following bacterial phagocytosis (Benoit et al. 2002). This resistance may result from its catalase activity. Although resistance was shown to be not a function of the virulence plasmid, treatment with hydrogen peroxide resulted in a fourfold and a sixfold enhancement, respectively, of *vapA* and *vapG* transcription (Benoit et al. 2002), supporting their role in survival under the oxidative conditions of the macrophages.

Lipids of the phagosome and membrane cholesterol are important carbon sources for intracellular pathogens. Beta-oxidation of lipids produces acetyl-CoA, which is further metabolized through the tricarboxylic acid cycle, after bypass of the two oxidative steps through the glyoxylate shunt (Kelly et al. 2002). The shunt involves conversion of isocitrate and acetyl-CoA into succinate and malate through the isocitrate lyase and malate synthase enzymes. The importance of membrane lipids as a source of carbon was confirmed by the marked virulence attenuation of isocitrate lyase mutants of *M. tuberculosis* (McKinney et al. 2000) and *Candida albicans* (Lorenz and Fink 2001). An isocitrate lyase (*aceA*) mutant of *R. equi* introduced intrabronchially was found to be completely attenuated in 3-week-old foals (Wall et al. 2005), confirming the crucial importance of β-oxidation of membrane lipid-derived fatty acids as a carbon source and their assimilation through the glyoxylate pathway. The *aceA* gene of *R. equi* is cotranscribed with the 3-hydroxyacyl-CoA dehydrogenase (*fadB*) gene involved in beta-oxidation of fatty acids but, as in *M. tuberculosis*, is not linked to the malate synthase gene (Kelly et al. 2002). The *aceA* transcripts are more abundant, probably because a hairpin structure between it and *fadB* protects it from exonuclease attack. A stable *aceA* transcript may provide the high isocitrate lyase activity required for successful competition for isocitrate between this glyoxylate shunt enzyme and isocitrate dehydrogenase (Kelly et al. 2002).

Transcriptional Regulation of the PAI and of Virulence

Two regulatory genes, *virR* (*orf4*), a LysR-type transcriptional regulator, and *orf8*, an orphan two-component regulator, are present on the PI. Dis-

ruption of either regulatory gene fully attenuates virulence (Ren and Prescott 2004). Russell et al. (2004) showed that *virR* is co-transcribed with four downstream genes (*orf5*, *vapH*, *orf7*, *orf8*) as a single message, thus constituting the *virR* operon. *VirR* is required for the full expression of *vapA* and to bind directly to the *vapA* promoter (Russell et al. 2004). However, it is also clear that *vapA* expression is controlled by *orf8*, the orphan two-component regulator gene, the sensor kinases for which have not yet been identified; *orf8* does not however regulate transcription of the *virR* operon (Byrne et al. 2007). Microarray analysis of PI gene expression under different growth conditions (Ren and Prescott 2003), however, suggested differential expression of genes within the *virR* operon described by Russell et al. (2004). Further analysis showed that v*irR* is transcribed constitutively at a low level, whereas the other four downstream genes are induced by low pH and high temperature. The difference in mRNA levels of the regulatory gene *virR* and the four downstream genes was not due to differential mRNA stability since the half-lives of these transcripts were the same. Unexpectedly, the four genes downstream of *virR* are transcribed both from the promoter of *virR* and from a second promoter, P_{orf5}, 585 bp downstream of the *virR* initiation codon (Byrne et al. 2007). Whereas VirR binds to a site overlapping the initiation codon of *virR*, resulting in negative autoregulation of the gene and explaining its low constitutive transcription, P_{orf5} is induced by high temperature and low pH, leading to increased transcription of the genes downstream from *virR*. In contrast to P_{virR} and P_{vapA}, P_{orf5} does not share similarity with *Mycobacterium* promoters that depend on the principal sigma. Therefore, Byrne et al. (2007) suggested that P_{orf5} is recognized by an alternative sigma factor, which adds another layer of complexity to PI gene regulation. The fact that the only two regulatory proteins encoded by the PI are located within a single operon suggested that this *virR* operon may act as a master switch of virulence gene regulation. In this model, low constitutive transcription of *virR* and *orf8* during saprophytic growth of the pathogen acts as a sentinel to determine the presence of a host; subsequent exposure to the host environment (increased temperature and reduced pH, among others) activates P_{orf5}, leading to increased levels of the response regulator Orf8, thus priming the expression of virulence genes such as *vapA*. Transcription of these virulence genes occurs once Orf8 is activated through its as yet unidentified cognate sensor kinase protein. The conservation of the *virR* operon in *vapA* (horse)- and *vapB* (pig)-type virulence plasmids supports the hypothesis that it plays a central role in regulation of virulence in *R. equi*. See Figure 9.2.

Although *vapA* was initially thought to be a monocistronic transcript, based on Northern blot analysis (Benoit et al. 2002; Russell et al. 2004), more sensitive RT-PCR analysis showed that it is co-transcribed as an operon with the downstream *vap*(*I*)*CD* genes (Byrne et al. 2008). Interestingly, the *vapA* mRNA is more abundant than that of the down-stream genes, which is due to processing of the *vapAICD* transcript. The initial *vapA* transcript has a half-life that is several times longer than those of *vapICD*. The increased half-life of *vapA* mRNA would lead to a more abundant synthesis of the VapA protein compared with VapC and D, which may reflect the different cellular localization and thus a different role and requirement for different levels of these proteins. VapA is tethered to the cell wall, whereas VapC and D are secreted into the external medium (Byrne et al. 2008). Intriguingly, the *vapCD* intergenic region contains two small Orfs that may encode very small proteins with signalling or other functions (Byrne et al. 2008).

Lesions

Pathological lesions correspond to the ability of the organism to replicate in macrophages and are characteristically granulomatous. Once macrophage destruction occurs through virulence plasmid-associated necrosis, lesions become pyogranulomatous. Although the pyogranulomatous lesions most typically occur in the lungs, tuberculosis-like granulomas, as noted, may occur in the submandibular lymph nodes of cattle and pigs. In foals, ulcerative lesions may be present in Peyer's patches in the large intestine, suggesting an ability of the pathogen to invade cells other than those of monocyte–macrophage origin.

Immunity and Its Impact on Pathogenesis

Immunity to *R. equi* pneumonia in foals likely depends on both the antibody and cell-mediated components of the immune system, but its exact basis remains to be determined. Whether or not pneumonia develops in foals exposed to virulent *R. equi* appears to be intimately linked to the subtleties

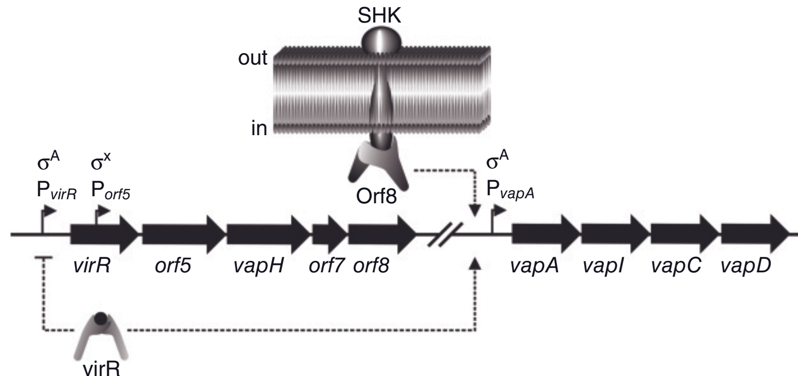

Figure 9.2. Schematic representation of the *virR* and *vapA* operons (not to scale) of the PAI and their transcriptional regulation by the LysR-type transcriptional regulator VirR and the response regulator Orf8. The promoter P_{virR} is constitutively active at a low level, which results in the transcription of the five genes of the *virR* operon. VirR binds to a site overlapping P_{virR} resulting in a negative autoregulatory circuit. An increase in temperature and decrease in pH of the growth medium activates P_{orf5}, resulting in increased transcription of *orf5*, *vapH*, *orf7*, and *orf8*. The latter is a response regulator that interacts with an as yet unidentified sensor histidine kinase (SHK) protein located in the cell membrane. VirR and presumaby Orf8 bind to the promoter of the *vapA* operon (P_{vapA}) and activate transcription. P_{vapA} and P_{virR} are similar to promoters that are recognized by the main vegetative sigma factor (σ^A) of *Streptomyces coelicolor* and *M. tuberculosis*, whereas Porf5 is not, indicating that activity of the latter promoter is dependent on an alternative sigma factor (σ^x).

of the developing immune system of the young foal, in ways that are not fully understood. The unique PI of the plasmid of foal-virulent *R. equi* may take advantage of an innate tendency of foals to respond to intracellular infections with an ineffective Th2- rather than an effective Th1-type immune response, although there is also evidence that possession of the virulence plasmid drives a Th2 rather than a Th1 response.

Antibodies to *R. equi* are widespread in horses. The age of development of *R. equi* pneumonia in foals coincides with and may be related to the decline in maternally derived antibodies. Evidence for a role of antibody in protection against *R. equi* is the protective effect of passively transferred anti-*R. equi* hyperimmune equine plasma, which is used extensively on endemically affected farms to reduce morbidity and mortality. Hooper-McGrevy et al. (2001) showed that purified VapA and VapC antibodies provided protection of foals against experimental pneumonic infection equivalent to that obtained with hyperimmune plasma. Interestingly, however, foals born to vaccinated mares are not protected against infection despite passive transfer of the anti-*R. equi* antibody. This failure may partly relate to the isotype of the antibody induced by the vaccines used experimentally but more likely relates to the crucial importance of an effective cell-mediated immune response in protecting foals against infection.

Almost all knowledge of cell-mediated immunity to *R. equi* infections comes from infection of mice. Because of the facultative intracellular nature of *R. equi*, cell-mediated immune mechanisms are thought to be of major importance in resistance. Key studies by Kanaly and coworkers (reviewed by Hines et al. 1997) have confirmed this in mice. For example, immunocompetent BALB/c mice experimentally infected with virulent *R. equi* developed a Th1 cytokine response and progressively cleared the infection. Mice in which a Th2 response was induced by administration of monoclonal antibodies against IFN-γ failed to clear the infection and developed pulmonary granulomas. Adoptive transfer of a Th1

cell line reactive against *R. equi* antigens into nude mice resulted in expression of IFN-γ mRNA locally in bronchial lymph node cells and clearance of *R. equi* from the lungs. In contrast, mice transfused with a *R. equi*-specific CD4+ Th2 cell line expressing IL-4 but not IFN-γ mRNA failed to clear pulmonary infection and developed large granulomas in the lungs. Together, these observations show that a Th1 cell-mediated immune response is absolutely required for effective clearance of *R. equi* in mice.

The two major mechanisms by which T lymphocytes mediate clearance of intracellular pathogens are secretion of cytokines and direct cytotoxicity (usually mediated by MHC class I restricted CD8+ T lymphocytes). Kanaly et al. (1996) conclusively demonstrated the central role of CD4+ T cells since MHC class I transgenic mice deficient in CD8+ T lymphocytes cleared virulent *R. equi* from the lungs, whereas infection persisted in MHC class II transgenic mice deficient in CD4+ T lymphocytes and led to the formation of granulomas. Adult immune horses showed marked lymphoproliferative responses to recombinant VapA following intrabronchial challenge with virulent *R. equi* (Hines et al. 2001), as well as a marked CD4+ and CD8+ T-lymphocyte response, suggesting that both subsets play a role in clearance of infection from the lung, reinforcing also earlier conclusions of the importance of VapA as an immunodominant antigen. Stimulated cells showed significant increases in antigen-specific IFN-γ but not in IL-4 expression, and local levels of IgGa and IgGb isotypes against VapA were dramatically enhanced after challenge, suggesting that these may be important correlates of protective immunity. Interestingly, IgGa and IgGb fix complement, whereas IgGT does not, and indeed IgGT may inhibit complement fixation by IgGa and IgGb (Lopez et al. 2002). Cytotoxic T lymphocytes from the lungs of 3-week-old foals were less able to lyse peripheral blood-adherent cells than those of older foals and adult horses (Patton et al. 2005). Patton et al. (2004) have shown a role for non-MHC class I restricted CD8+ cytotoxic T lymphocytes in immunity of adult horses to *R. equi* antigens other than VapA, suggesting that CD1 or other nonclassical MHC molecules may also be important in antigen processing, potentially including antigens such as ReqLAM. Pargass et al. (2009) observed reduced expression of both CD1 and MHC class II molecules on monocyte-derived macrophages from foals compared with adults, and suggested that this might explain the unique susceptibility of the foal. The down-regulation of CD1b observed in equine monocyte-derived macrophages following infection with *R. equi* may represent a novel mechanism by which *R. equi* avoids detection and killing by the immune system (Pargass et al. 2009).

It is apparent that infection of foals by virulent *R. equi* can result in an immunomodulatory effect that drives an ineffective, Th2-like, rather than an effective, Th1-like, response. For example, Giguère et al. (1999) found that foals experimentally infected with a virulent *R. equi* strain showed marked reduction in IFN-γ production by CD4+ lymphocytes isolated from bronchial lymph nodes compared with CD4+ lymphocytes similarly isolated from foals infected with an avirulent, plasmid-cured derivative of the same strain, and that concentrations of IL-10, a Th1-immune response down-regulatory cytokine, were significantly higher in the lungs of foals infected with the virulent strain. The conclusion was that virulent *R. equi* have an immunomodulating effect that drives an ineffective, Th2-like immune response. Interestingly, Hines et al. (2003) observed that clearance of virulent *R. equi* from the lung of experimentally infected adult horses was associated with increased numbers of IFN-γ producing CD4+ and CD8+ lymphocytes. This difference between immune adults and nonimmune foals may reflect unique features of the foal's immune system, differences between immune and nonimmune animals, or differences in the experimental procedures including the challenge dose.

Immunization of foals under natural settings with a VapA extract in aluminum hydroxide adjuvant resulted in development of pneumonia in immunized but not in nonimmunized foals, and an IgGb- and IgGT-dominated isotype response compared with an IgGa response in immune foals (Prescott et al. 1997). Hooper-McGrevy et al. (2003) also observed an IgGb- and IgGT-dominant response to Vap proteins in the serum of foals sick with *R. equi* compared with healthy, immune foals and adults, which had an IgGa-dominant response. Both these studies were interpreted as supporting the concept that foals that become sick with *R. equi* do so because of an ineffective, Th2-dominated immune response. Because of the reciprocal relationship between these two types of immune response or perhaps also because of an interference effect of IgGT on complement activation by IgGa, immunization with VapA in an adjuvant that drives a Th2

response would be expected to result in development of disease. Interestingly, infection of 1-week-old foals with an *aceA-choE* mutant resulted in delayed development of severe mutant-induced pneumonia in two of five foals but in no pneumonia in the other three foals (Pei et al. 2007b), suggesting that the ability to mount an effective immune response, which may be age related, are important in determining the attenuation. In *M. tuberculosis*, attenuation of the aceA mutation is dependent on a functioning cell-mediated immune system (McKinney et al. 2000).

Current evidence therefore suggests that infection of foals with virulent *R. equi* may result in subversion of cell-mediated immunity and development of an ineffective and sometimes lethal immune response. What factors determine this outcome? Although details are not clear, the PI on the virulence plasmid must play a crucial role. The number of bacteria inhaled by foals is almost certainly also important. For example, the dose of Bacille Calmette-Guérin (BCG) used to immunize mice determined whether Th1 or Th2 responses developed, with low doses inducing an almost exclusive cell-mediated, Th1 response and higher doses producing a mixed Th1/Th2 response (Power et al. 1998). A similar effect may be occurring in *R. equi*. The presence and probably the isotype of maternally derived immunoglobulin are also significant. Other factors predisposing foals to a Th2-like immune response may also be important. For example, European workers have suggested that equine herpesvirus type 2 (EHV-2) predisposes to *R. equi* bronchopneumonia since immunization of foals against EHV-2 appeared to control *R. equi* pneumonia (Nordengrahn et al. 1996). Interestingly, EHV-2 possesses an IL-10 gene homologue, which could be speculated to be involved in immunomodulation in foals.

Other factors that may appear to be important in determining an ineffective immune response of foals to *R. equi* include a postulated tendency of young foals to be IFN-γ deficient and to be Th2 polarized. The evidence for this is conflicting, with some evidence showing a general age-related IFN-γ deficiency (Breathnach et al. 2006), whereas other evidence shows that bronchial lymph node cells of young foals can respond well with an IFN-gamma response to *R. equi* infection, although naïve foals responded poorly to *R. equi* antigens in lymphoproliferative assays (Jacks et al. 2007a). By contrast, young foals were as immunocompetent as adults in recall responses to Vap antigens (Jacks et al. 2007b). Dendritic cells are crucially involved as antigen-presenting cells in the innate immune response to infection and in determining the nature of the immune response. Studies of the interaction over time of foal monocyte-derived dendritic cells with *R. equi* showed that, although the expressions of co-stimulatory molecules CD40 and CD86, as well as the cytokine IL-12 and transcriptional factor IRF-1, were similar between foals and adult horses, in foals they were not accompanied by robust MHC class II molecule expression (Flaminio et al. 2008), which may compromise efficient priming of effector cells. Others have shown that neonatal foals have an apparent selective impairment of production of some cytokines (IFN-γ, IL-6) but not of others (IL-8, IL-12, IL-23) in peripheral blood mononuclear cells in response to *R. equi* stimulation (Liu et al. 2008). Interestingly, cells of the monocytic lineage, which includes dendritic cells and macrophages, from foals responded to IFN-γ stimulation by greater production of the immunomodulatory cytokine IL-10 than similar cells from adult horses (Sponseller et al. 2008). In summary, besides "naivity" of the neonatal immune system because of lack of exposure to microbial antigens before birth, there may be age-related impairments both in important antigen-presenting molecules and in cytokines important for effective cell-mediated immune responses, which synergize with the pathogen in driving an ineffective immune response, thus allowing the successful continued replication of *R. equi* in macrophages and its spread from the lungs back into the environment, either by aerosol or through the intestine following swallowing of infected sputum. The topic has important clinical implications since the impaired cytokines can be increased by CpG-ODN for therapy, at least *in vitro* (Liu et al. 2008).

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

The availability of the completed genome of *R. equi* is a major advance in the field, opening the field for microarray analysis of virulence regulons, when combined with a reliable counterselectable mutation system and the ability to produce stable site-specific integration. Improved understanding of the nature of the plasmid PI through recent analysis of the vapB-type plasmids is focusing effort to understand the role of the PI in subverting development of the phagosome and of the foals' protective immune

response. The extraordinary explosion of understanding of *M. tuberculosis* as a pathogen both serves as a model for understanding the complexity of *R. equi* and shows how understanding of *R. equi* as a pathogen can potentially be used to improve control of human tuberculosis. Although there are many gaps in understanding, there is potential for considerable advances in the future.

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10
Listeria

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INTRODUCTION

The six species in the genus *Listeria* are *Listeria monocytogenes, Listeria ivanovii, Listeria innocua, Listeria seeligeri, Listeria Welshimeri*, and *Listeria grayi*. Results of genomic analysis suggest that these species fall into three main groups. The first contains *L. monocytogenes, L. innocua*, and *L. welshimeri*, the second group *L. ivanovii* and *L. seeligeri*, and the third the more distantly related *L. grayi* (Hain et al. 2007). Multiple gene acquisitions and deletion events are evident among these species. For example, comparison of *L. monocytogenes* strain EGDe (serotype 1/2a) and *L. monocytogenes* strain F2365 (serotype 4b) revealed gene reduction in the latter, suggesting derivation of serotype 4b from serotype 1/2 (Hain et al. 2007). The encoded proteins of *L. monocytogenes* have considerable similarity with those of *Bacillus subtilis* (Cabanes et al. 2002). Proteomic analysis reveals differences in protein expression between *L. monocytogenes* strains F2365 and EGD (Donaldson et al. 2009).

Of these, only *L. monocytogenes* and *L. ivanovii* are pathogenic. The former is responsible for the vast majority of cases, causing septicemia, abortion, and central nervous system (CNS) infection in a wide range of animal species, including humans. *L. ivanovii* shares certain characteristics with *L. monocytogenes* (e.g., hemolysis) and is occasionally associated with abortion in ruminants (especially sheep). However, it does not cause CNS infection and is not a pathogen of significance in other animal species, including humans. The remaining four *Listeria* species are considered avirulent and will not be considered further.

A multitude of subtyping schemes suggest that *L. monocytogenes* is partitioned into three major lineages (reviewed in Kathariou 2002; Cheng et al. 2008). Lineage I contains strains of serotypes 1/2b, 3b, and 4b, and lineage II includes strains of serotypes 1/2a, 3a, 1/c, and 3c. Lineage III includes strains of serotypes 4a and 4c, as well as certain strains of serotype 4b, and may contain more isolates from animal listeriosis than from human disease (Roberts et al. 2006; Liu et al. 2006; Ducey et al. 2007; Ward et al. 2008). Animal isolates can also be found in lineages I and II.

Veterinary medical concern with *L. monocytogenes* is focused primarily on disease in cattle and sheep. In addition, it is a significant opportunistic foodborne pathogen of humans, often with devastating results (Farber and Peterkin 1991; Schuchat et al. 1991; Farber et al. 1996; Kathariou 2002). The reported incidence of human cases is low compared with that for other foodborne bacterial pathogens, such as *Campylobacter* and *Salmonella*, but the mortality rate in human listeriosis is the highest among human foodborne bacterial pathogens (an estimated 2500 cases and 500 deaths per year in the United States) (Notermans et al. 1998; Hurd et al. 2000). As such, veterinarians must be aware of both the impact of *L. monocytogenes* on infected ruminants and the public health concerns surrounding the entry of *Listeria*-contaminated products of animal origin, especially milk and dairy products, into the human food chain (fig. 10.1).

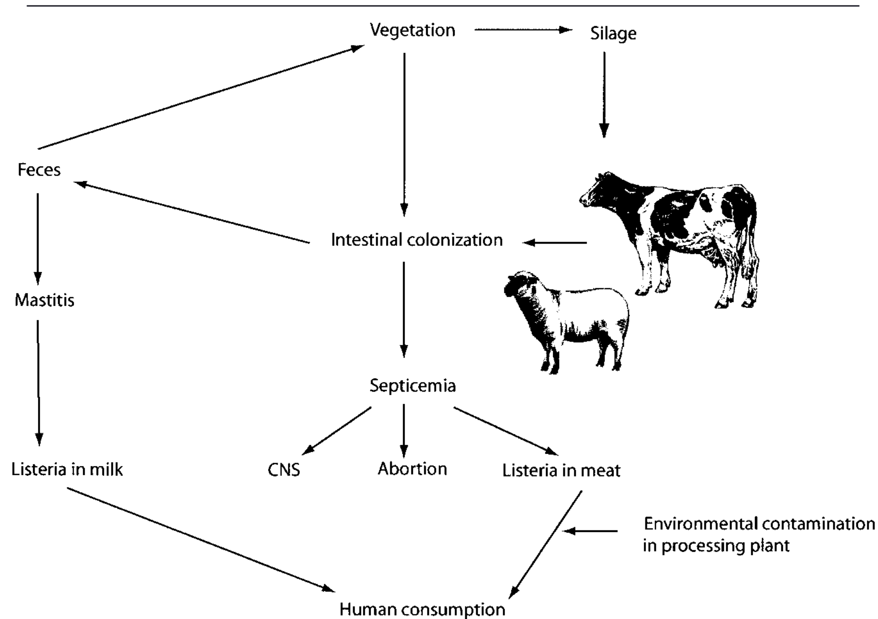

Figure 10.1. Overview of listeriosis in ruminants and possible route of introduction into food products for human consumption.

CHARACTERISTICS

Listeria monocytogenes can multiply in diverse environmental conditions, as might be expected for a bacterium capable of free-living growth. It is relatively resistant to NaCl, and growth is possible at pH 5–9. The latter is a root cause of some outbreaks of listeriosis in ruminants (i.e., poorly prepared silage that does not achieve appropriate acidity) (Low and Renton 1985; Fenlon 1986). Perhaps most important is its ability to grow at temperatures from 1°C to 45°C. Growth (albeit slow growth) at low temperatures has been exploited by both the organism and laboratory diagnosticians. *L. monocytogenes* can multiply to high numbers in silage (Fenlon 1986) or in food products maintained at room or refrigeration temperatures (Aureli et al. 2000; Farber and Peterkin 1991). This poses a threat to the animal or person that ingests the contaminated material and presents a mechanism for selection and enrichment for listeriae (i.e., cold enrichment) in clinical or environmental specimens prior to the availability of selective media. Recent evidence suggests that exposure to low temperatures triggers in *L. monocytogenes* a cold stress response that is both dependent on and independent of sigmaB (reviewed in Tasara and Stephan 2006; Chen et al. 2007) and is mediated in part by a family of proteins that also contribute to salt tolerance (Schmid et al. 2009).

Listeria monocytogenes forms small translucent colonies in 24–48 h when incubated aerobically or anaerobically at 37°C on common bacteriological media, such as trypticase soy agar or brain heart infusion agar. The organism is hemolytic, although the zone of hemolysis can be narrow. *L. monocytogenes* produces a CAMP reaction with a hemolysin of *Staphylococcus aureus*. In contrast, *L. ivanovii* typically produces double-zone hemolysis and is negative in the CAMP reaction (Schuchat et al. 1991). Gram staining reveals thin, non-spore-forming gram-positive rods of variable length. The organism is oxidase negative, catalase positive, H_2S negative, and motile when cultivated at room temperature but not at 37°C. The latter reflects production of peritrichous flagella during growth at 25°C or lower, whereas flagella are rare when growth is at human body temperature (35–39°C). The emergence of *L. monocytogenes* as a foodborne pathogen has led to improvements in selective media (e.g., modified Oxford agar). Examples of newer selec-

tive/differential media include chromogenic media that detect phospholipase activity of *Listeria* spp. (Reissbrodt 2004; Hegde et al. 2007). Other detection methods include antibody-based tests and broth enrichment followed by detection via PCR or DNA probes (Zhao and Doyle 2001).

Thirteen serotypes of *L. monocytogenes* are based on somatic and flagellar antigens, with nearly all cases of animal and human infections being caused by serotypes 1/2a, 1/2b, and 4b (Gudding et al. 1989; Farber and Peterkin 1991; Schuchat et al. 1991). It is interesting to note that nearly all large outbreaks of human listeriosis have been associated with serotype 4b, whereas sporadic cases can be associated with other serotype 4b strains, as well as other serotypes, especially 1/2a and 1/2b (Notermans et al. 1998; Aureli et al. 2000; Hurd et al. 2000). Several epidemic clones (ECs) of serotype 4b have been recognized, including ECs ECI, ECII, ECIa (also designated ECIV), and ECV (Kathariou 2002; Ducey et al. 2007; Cheng et al. 2008). Outbreaks involving bacteria of serotype 1/2a have been less frequent; it was involved in the first recognized outbreak of human listeriosis (the Halle outbreak in Germany) (Reiss et al. 1951) and in a multistate outbreak of listeriosis, attributed to contaminated turkey deli meats in 2000 in the United States, with the corresponding strain designated ECIII (Kathariou 2002). Strains of serotype 1/2a and 1/2b have also been implicated in several outbreaks of febrile gastroenteritis (Kathariou 2002).

Predominance of a relatively small number of clonal groups among outbreak-associated cases remains unexplained but might reflect the greater ability of the corresponding strains to persistently contaminate processing plant environments and to replicate in ready-to-eat (RTE) foods. For instance, analysis of serotype 4b strains from foods revealed that approximately 40% of the isolates were members of ECI, implicated in several outbreaks in Europe and North America (Yildirim et al. 2004). Members of ECI and ECII were also highly represented among serotype 4b isolates from the environment of two turkey-processing plants in the United States (Eifert et al. 2005). Strains from the multistate outbreaks of listeriosis in 1998–1999, 2000, and 2002 harbor cadmium resistance, and those from the first two of these are also resistant to the quaternary ammonium disinfectant benzalkonium chloride (Elhanafi and Kathariou 2007; R. M. Siletzky and S. Kathariou, unpublished). Bacteria of ECII are resistant to phage when grown at temperatures below 30°C, suggesting a possible fitness advantage for this clonal group in the processing plant or in foods (Kim et al. 2009). It is also possible that bacteria in the epidemic clonal groups are better equipped to survive in the gastrointestinal (GI) tract and to translocate across the intestinal mucosa to establish systemic infection (Barbour et al. 2001; Czuprynski et al. 2002).

Currently, we lack consistent evidence that EC strains have higher virulence potential than strains from sporadic cases (Kathariou 2002). On the other hand, strains of serotype 4b produce intact, full-length internalin protein, regardless of their source; food-derived strains of serogroup 1/2 frequently harbor premature stop codons in the internalin gene, *inlA*, resulting in truncated versions of the protein that cannot be anchored to the cell wall (Jacquet et al. 2004; Nightingale et al. 2008; Ward et al. 2008). Such strains may be less able to interact with the intestinal epithelium, and thus may be impaired in their ability to cause invasive illness following consumption of contaminated foods. Furthermore, serotype 4b strains harbor serotype-specific glycosylations of teichoic acid, which appear to be required for virulence after oral inoculation of mice (Faith et al. 2008). Nonetheless, such attributes are shared by all strains of serotype 4b, and thus are unlikely to underlie the prevalence of specific clonal groups of this serotype. Recently available genome sequence data of serotype 4b strains representing ECI and ECII have revealed genes and gene cassettes that appear to be unique to these clonal groups (Nelson et al. 2004). Future studies will determine whether such unique determinants contribute to special adaptations of the organisms, including possibly greater virulence for humans.

SOURCES OF INFECTION

Listeria monocytogenes is widely distributed in nature, having been isolated from soil, vegetation, water, feces, and a variety of vertebrate and invertebrate species (Gray and Killinger, 1966). The organism is hardy and persistent in the environment. In particular, *Listeria* spp. can be isolated from drains and other surfaces in food-processing facilities even when standard hygienic practices are in place (Farber et al. 1996; Notermans et al. 1998; Aureli et al. 2000; Kells and Gilmour 2004). The sources of the bacteria that colonize the processing plant environment remain largely unknown, as are reservoirs of *Listeria* in nature. The ubiquity and adaptations of these bacteria suggest that their

reservoirs may be diverse and complex. Animals (especially ruminants) may constitute a reservoir that ultimately contributes to periodic bacterial introductions into the processing plant environment. This could occur directly through animals entering the facilities for slaughter and processing or indirectly through human traffic and other vehicles. Fecal shedding of *L. monocytogenes* by dairy cattle is common, in numbers and serotypes that are quite variable (Boruckim et al. 2005; Ho et al. 2007). *L. monocytogenes* also remains viable for weeks in manure or in soil on which manure has been spread (Jiang et al. 2004; Nicholson et al. 2005). The potential for contamination of silage on a farm, or exposure of animals to *L. monocytogenes* in manure, is likely significant. Contamination of surface water downstream of farms is also a possibility (Lyautey et al. 2007).

VIRULENCE FACTORS

Listeria monocytogenes has served as an exemplary model for intracellular parasitism. Work from several laboratories has elucidated a complex interplay among virulence factors that are involved in attachment, internalization, and survival and multiplication in the cytoplasm of infected cells (Decatur and Portnoy 2000; Cabanes et al. 2002; Lecuit et al. 2001). This work has been done principally in cell monolayers and mouse infection models, but the general principles delineated are also likely to be operative in *L. monocytogenes* infections of ruminants and humans.

As illustrated in the simplified overview in fig. 10.2, *L. monocytogenes* invades both professional and nonprofessional phagocytes via the activity of internalin, a family of surface proteins covalently linked to the cell wall (A) (Gregory et al. 1997; Cabanes et al. 2002; Lecuit et al. 2001). The internalized listeriae then escape from the phagosome via the action of their hemolysin (listeriolysin O [LLO]) and a phosphatidylinositol-specific phospholipase C (B) (Schlüer et al. 1998; Decatur and Portnoy 2000; Glomski et al. 2002; Kohda et al. 2002). The listeriae multiply in the cytoplasm with an estimated doubling time of ~1 h (C). The bacterial protein ActA directs the nucleation of host-cell-derived actin filaments onto one end of each organism (Beckerle 1998; Darji et al. 1998; Moors et al. 1999). The continued addition of F-actin monomers propels the bacilli through the cytoplasm and toward the cell's periphery (D). When the organism presses against the interior surface of the cell membrane, projections (called listeriopods) invaginate into adjacent cells, allowing the listeriae to enter those

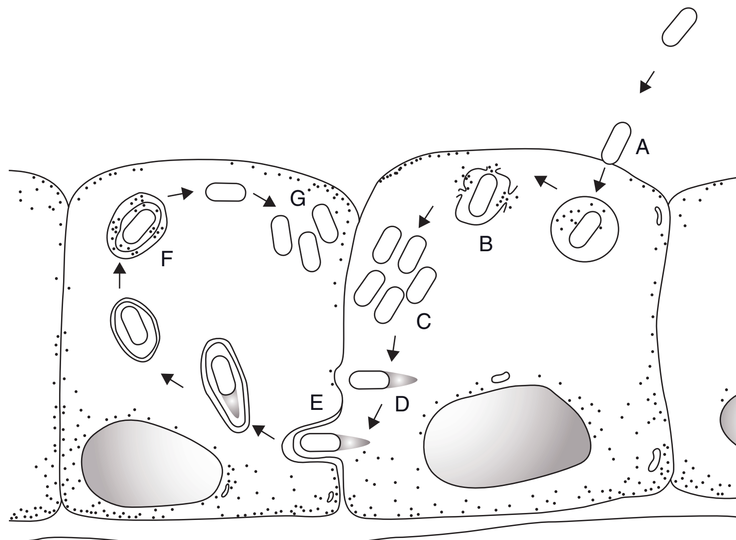

Figure 10.2. Simplified illustration of the events involved in the invasion, intracellular growth and cell-to-cell spread of *L. monocytogenes*.

cells in a double-membraned vacuole (E). The listeriae then escape from this vacuole via the action of the hemolysin and a phosphatidylcholine-dependent phospholipase C (lecithinase) (F) (Portnoy et al. 1989; Schlűter et al. 1998), and the process of intracellular multiplication is repeated (G). The series of events allows *L. monocytogenes* to multiply and spread without direct exposure to the cells and soluble factors of the extracellular milieu. Centripetal spread of organisms within foci of infection can sometimes be dramatically visualized via gram staining of lesions (Havel 1989; Gregory and Wing 2002).

Several genes responsible for these intracellular events have been identified, cloned, and sequenced. The *L. monocytogenes* genome is particularly rich in genes that encode surface proteins (nearly 5% of the genome) (Glaser et al. 2001; Cabanes et al. 2002). As noted previously, invasion involves at least two members of the internalin (Inl) protein family. InlA is an 80-kDa surface protein that is covalently linked to peptidoglycan by its carboxy-terminal domain (LPXTG). Similar LPXTG motifs are found in other gram-positive pathogens, including *S. aureus* and *Streptococcus pyogenes* (Cabanes et al. 2002). InlA binds to E-cadherin, which can be found on the basolateral surface of intestinal epithelial cells. Results of an elegant series of experiments (Lecuit et al. 2001) show that this interaction is of paramount importance in intestinal epithelial cell invasion and systemic infection via the GI tract. Other surface proteins, including InlB, another member of the internalin family, also participate in this process (Gregory et al. 1997; Lecuit et al. 2001). Interestingly, nonpathogenic *L. innocua* lacks more than 20% of the surface proteins (including InlB) encoded by *L. monocytogenes* (Cabanes et al. 2002; Glaser et al. 2001).

As noted, cholesterol-binding, thiol-activated hemolysin LLO and a phosphatidylinositol-dependent phospholipase C lyse the phagosome membrane and allow the bacteria to escape into the cytoplasm (Schlűter et al. 1998; Decatur and Portnoy 2000). LLO is more active at the acidic intraphagosomal pH (Glomski et al. 2002). A proline, glutamine, serine, and threonine (PEST) amino acid sequence targets LLO for intracytoplasmic degradation, thus reducing toxicity to the host cell (Decatur and Portnoy 2000). This ingenious mechanism undoubtedly contributes to the survival of infected cells *in vivo* and *in vitro* in spite of an immense listerial burden. Different domains of LLO are involved in lytic and stimulatory activities for mammalian cells (Kohda et al. 2002).

ActA is a 90-kDa surface protein that directs the host-dependent arrangement of actin filaments that propel the listeriae through the cytoplasm (Kocks et al. 1992). Expression of ActA is enhanced in the intracellular environment or by exposure to cell lysates (Darji et al. 1998; Moors et al. 1999; Shetron-Rama et al. 2002). Mutants that lack ActA can multiply intracellularly but are unable to invade adjacent cells. ActA and its interaction with the actin cytoskeleton have provided a powerful tool for understanding regulation of cell shape and motility (Beckerle 1998). ActA is recognized by the immune system during infection but is not a target for protective immunity, likely because of the intracellular location of the antigen (Darji et al. 1998).

Positive regulatory factor (PrfA) is responsible for coordinate regulation of the virulence factors of *L. monocytogenes* and is itself temperature controlled (Renzoni et al. 1999). The *prfA* expression is influenced by environmental cues (e.g., temperature, pH, nutrients); it is enhanced during infection of mammalian cells, beginning with the process of cell adhesion. *prfA* and related virulence genes are substantially upregulated during growth in human blood (as compared with growth in brain heart infusion broth or in the mouse intestinal tract) (Toledo-Arana et al. 2009). This supports a conclusion that *prfA* regulation is a key event in the expression of virulence determinants by *L. monocytogenes* after the bacteria have left the GI tract and invaded host tissues. Consistent with this hypothesis is the knowledge that the nonpathogenic *L. innocua* does not contain *prfA* (Glaser et al. 2001). The situation differs within the GI tract, where *prfA* seems to have a lesser role. In contrast, *sigB* and its corresponding regulon are required for survival in the gut (Faith et al. 2005; Garner et al. 2006; Toledo-Arana et al. 2009).

Mammalian cells are not simply passive targets in the invasion and intracellular multiplication of *L. monocytogenes*. Interaction of ActA with components of the actin cytoskeleton is well recognized, as described above. In addition, LLO activates MEK-1 and ERK-2 of the MAP kinase pathway (Tang et al. 1998) to induce NF-κB signaling by activating the IκB kinase complex that phosphorylates (and inactivates) IκB, a natural inhibitor of NF- κB (Kayal et al. 2002). InlB also activates

NF- κB, in this case via activation of the signaling molecules Ras, phosphoinositide 3-kinase, and Akt (Mansell et al. 2001).

The studies that have provided the information described above represent an elegant marriage of contemporary molecular biology with immunology and traditional cell biology. What is particularly satisfying is the manner in which they provide a means of synthesizing and explaining earlier descriptive observations of the ability of *L. monocytogenes* to invade epithelial cells and neuronal cells *in vivo* (Asahi et al. 1957; Charlton and Garcia 1977).

PATHOGENESIS

Disease in Ruminants

Listeria monocytogenes is an infrequent but serious pathogen of ruminants (both domestic and exotic) (Gray and Killinger 1966). In the United States, greatest attention is placed on infections of dairy and beef cattle. In other parts of the world, listeriosis in sheep and goats assumes greater importance. In both cattle and sheep, listeriosis can manifest itself as (1) a CNS infection (meningoencephalitis in adults and meningitis in the young), (2) abortion, (3) generalized septicemia involving the liver and other organs, and (4) mastitis. Affected animals tend to present with only one of these clinical syndromes. For example, listeriosis may present as a cluster of abortions or as neurologic disease in one or more individual animals.

Listeriosis of the CNS is a common presentation that must be included in the differential diagnosis of ruminants with neurologic changes. It is an unexplained curiosity that meningoencephalitis is seen in adult ruminants, whereas CNS infection typically presents as meningitis without brain involvement in monogastric species and in young ruminants before the rumen becomes functional (Gray and Killinger 1966). The clinical presentation of meningoencephalitis in adult ruminants may begin with signs of depression and confusion (Low and Renton 1985; West and Obwolo 1987). The ears droop, and the animal holds its head to one side. Protrusion of the tongue and salivation are common, and twitching or paralysis of the facial and throat muscles may occur. The animal may lean against fences or other objects for support (fig. 10.3). When the animal moves, it tends to be in a circle, either clockwise or anticlockwise, giving rise to the common name of "circling disease." In the terminal stages, the animal may fall and be unable to rise; tremors are common.

Listeria monocytogenes is seldom recovered from the cerebrospinal fluid of ruminants with meningoencephalitis, contrary to findings in human meningitis patients. Diagnosis is based on clinical signs and a finding of cerebrospinal mononuclear pleocytosis (Schweizer et al. 2006). Spontaneous recovery from severe clinical disease is rare and may be accompanied by permanent neurologic damage. Published reports (Schweizer et al. 2006) and clinical experience at the University of Wisconsin-Madison Veterinary Medical Teaching Hospital indicate that antimicrobial therapy is most effective if started early in the disease process. Such therapy can be a challenge due to limited drug bioavailability in the CNS, poor penetration of drug into brainstem microabscesses, and the limited number of drugs that can be used in food animals. Recommended antimicrobials include oxytetracycline or penicillin, administered for a minimum of 10 days or 14–21 days for more severe cases (Rebhun and de Lahunta 1982). Supportive therapy may also be indicated and could include bicarbonate and potassium to replace losses due to excessive salivation when there is poor cranial nerve function. Nursing care and good footing for the affected cow are important because affected animals are frequently ataxic and can become recumbent (Schweizer et al. 2006).

The pathogenesis of meningoencephalitis in ruminants is only partially understood. In the past, a definite seasonal association was noted, with disease being more common in winter or early spring when the animals are housed indoors rather than on pasture. Although this is still observed in some settings (Nightingale et al. 2005), seasonality becomes less important as dairy cattle spend less time on pasture and are managed more intensively in freestall barns. There is also a strong association between occurrence of listeriosis and feeding of silage rather than hay (Barlow and McGorum 1985; Low and Renton 1985; Gudding et al. 1989). The ability of *L. monocytogenes* to multiply in poor quality silage ($pH > 5.0$) is well documented (Fenlon 1986); *L. monocytogenes* strains isolated from affected cattle and sheep are often of the same ribotype as those from silage the animals consumed (Wiedmann et al. 1997).

It is not clear how *L. monocytogenes* breaches the GI epithelial barrier. Radiolabeled *L. monocytogenes* was used to follow bacterial passage through

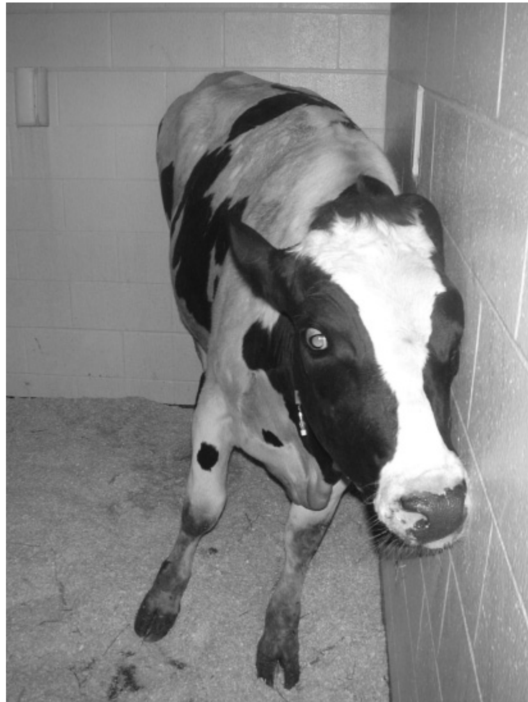

Figure 10.3. A 14-month-old Holstein heifer with *Listeria* meningoencephalitis ("circling disease"). Note the characteristic head tilt, ear droop, and leaning against the wall. The heifer circled to the right when induced to walk, and leaned on walls when standing. This animal was treated and survived. (See color plate)

the GI tract and dissemination to the reticuloendothelial system of sheep (Zundel and Bernard 2006). Listeriae were distributed throughout the GI tract within 24 h and were shed in feces for up to 10 days. Translocation to the spleen, liver, and lymph nodes was also noted, as were substantial numbers of listeriae in the palatine tonsils (Zundel and Bernard 2006). Listeriosis in sheep may result when *Listeria* penetrates through the dental pulp when sheep are cutting or losing teeth (Barlow and McGorum 1985). Listeriosis in goats foraging on hard, woody plants also suggests transmission through abrasions in the oral cavity (Johnson et al. 1996). There is older, but intriguing, evidence that once *L. monocytogenes* leaves the oral cavity, it invades the trigeminal nerves and travels centripetally along the axons to the brain (Asahi et al. 1957; Charlton and Garcia 1977). Observations of spontaneous disease in cattle (West and Obwolo 1987) are consistent with this route of invasion. In addition, morphologic evidence for a similar progression in the absence of septicemia was presented for mice and rabbits inoculated in the lips with *L. monocytogenes* (Asahi et al. 1957). The possibility that *L. monocytogenes* directly spreads along nerves is more easily accepted in light of contemporary knowledge of the organism's ability to invade and spread directly from cell to cell.

Gross morphology of the brains of ruminants suffering from encephalitis may appear normal, or there may be focal microabscessation and hemorrhage (fig. 10.4). Typical microscopic lesions are perivascular cuffing of mononuclear cells and occasional inflammatory foci (microabscesses) that contain both granulocytes and mononuclear cells. These lesions are most common in the midbrain, pons, and medulla oblongata (Asahi et al. 1957; Charlton and Garcia 1977). Examination of gram-stained tissue sections may reveal listeriae in large lesions, but they are infrequently observed in the smaller perivascular cuffs. Inducible nitric oxide synthase is observed in abscesses but not in

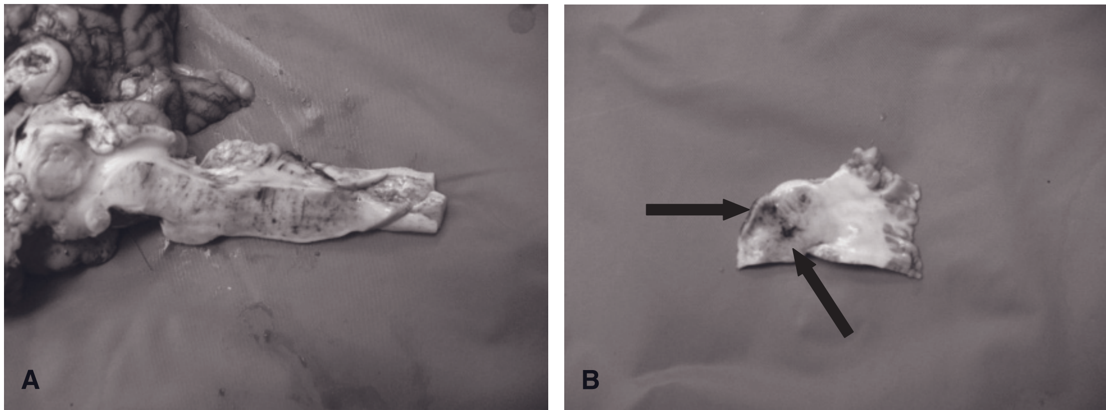

Figure 10.4. Sagitial section (A) and transverse section (B) of the brainstem of a 3-year-old Holstein cow that presented for neurologic dysfunction. Note the micro-abscessation associated with hemorrhages in the tissue (arrows). (See color plate)

perivascular cuffs of mononuclear cells in the brains of infected cattle; the reverse relationship is observed for expression of MHC class 2 antigens in the brains of infected cattle (Jungi et al. 1997; Shin et al. 2000). Results of studies with laboratory animals suggest that CNS invasion may be facilitated by extravasation of *Listeria*-infected blood monocytes (Drevets 1999).

Listeria monocytogenes may have a predilection for fetoplacental invasion in humans and a variety of other mammals (Gray and Killinger 1966). Abortion typically occurs in the third trimester of pregnancy and may result in obvious clinical disease in the dam (Gray and Killinger 1966). Abortion and other reproductive problems occurring in association with septicemia and encephalitis have been reported in sheep (Vasquez-Boland 1992). These outbreaks can result in substantial economic losses (Low and Renton 1985; reviewed by Wesley 2007). The organism can be demonstrated microscopically or by bacteriologic culture in the aborted fetus or placenta of affected sheep (Low and Renton 1985).

Results of experimental work in mice suggest that T cells and macrophages do not reach the infected placenta in sufficient numbers to be protective (Redline et al. 1988). A role for internalin (*inlA*) in invasion of trophoblasts and infection of the fetus seems certain (Lecuit et al. 2001; Disson et al. 2009). Other investigators report the importance of ActA in mouse and guinea pig fetal infection (Bakardjiev et al. 2005; Le Monnier 2007). Dose-response studies in both guinea pig and rhesus monkey models suggest that 10^7 CFU will cause fetal infection in ~50% of pregnant animals (Williams et al. 2007; Smith et al. 2008).

Listeria monocytogenes has also been associated with generalized septicemia and focal necrosis of the spleen and liver in various ruminants (Gray and Killinger 1966; Low and Renton 1985). Considerable attention has been paid to the problem of mastitis in dairy cattle (Gitter 1980), which can range in severity from subclinical to severe suppurative infection. Bovine feces (Weber et al. 1995) and the dairy farm environment (Ueno et al. 1996) are sources of the organism for mammary infections. Serotype 1/2a and serotype 4b strains cause experimental mastitis in cattle (Bourry et al. 1995), with similar susceptibility in sheep (Bourry et al. 1995; Tzora et al. 1998). *L. monocytogenes* can be found within neutrophils in milk from mastitic cows (Doyle et al. 1987), but speculation that this intracellular localization protects listeriae from effects of short-term pasteurization has been proven to be unfounded (Bunning et al. 1988). Long-term infection of the udder may ensue, with shedding of listeriae in milk exacerbated by periodic immunosuppression (Wesley et al. 1989). This has practical implications since successful treatment of *L. monocytogenes* mastitis is difficult to achieve (Gitter 1980). Longitudinal analysis revealed repeated positive samples from milk lines and bulk tanks, with more than one pulsed-field gel electrophoresis type of *L.*

monocytogenes detected (Ho et al. 2007). In an intriguing recent report, staphylococcal phages transduced staphylococcal pathogenicity islands harboring superantigen-encoding genes into *L. monocytogenes* when both *L. monocytogenes* and *S. aureus* were present in raw milk (Chen and Novick 2009). Thus, coinfection of the udder with both pathogens, especially when phages are present (naturally or as biocontrols for *S. aureus*), may have unexpected consequences.

Listeriosis in Nonruminant Animal Species

Clinical listeriosis is rare in horses, pigs, dogs, and cats. Healthy pigs may occasionally excrete *L. monocytogenes* in feces, but bacteria were recovered primarily from the tonsils of slaughtered pigs (Autio et al. 2000; Wesley et al. 2008). Recovery from pig carcasses was infrequent (Wesley et al. 2008), and such contamination may result from transfer of the bacteria from the processing plant environment rather than fecal contamination through slaughter and processing. The apparent low prevalence of *L. monocytogenes* and listeriosis in pigs (Wesley 2007; Wesley et al. 2008) suggests that disease is of limited economic significance (Leman et al. 1986).

Septicemic listeriosis in dogs and cats is extremely rare (Greene and Prescott 1998), although generalized infection and meningitis were reported in a Doberman (Schroeder and van Rensburg 1993). Cats infected via subcutaneous injection develop a strong delayed-type hypersensitivity response (Pedersen et al. 1998). Infection is severe in feline immunodeficiency virus (FIV)-infected cats (Dean et al. 1998). Serotype 4 was recovered from a gray fox and a raccoon dog (*Nyctereutes procyonoides*, a canid indigenous to China, Korea, Japan, and parts of Siberia) with microscopic lesions of canine distemper (Black et al. 1996; Aoyagi et al. 2000). The organism has been isolated from feces of deer, moose, otter, and raccoon (Lyautey et al. 2007).

Earliest descriptions of *L. monocytogenes* infections often involved guinea pigs, rabbits, and gerbils (Seeliger and Jones 1986), and laboratory rodents have served as the primary models for the study of listeriosis (Kathariou 2002). Gerbils have now been proposed as a biologically relevant model for orally transmitted listeric infections due to their possession of receptors for both major internalins (Disson et al. 2009). The prevalence of spontaneous listeriosis in small rodents kept as pets is not known, but veterinarians whose practices include pocket pets might keep listeriosis in mind as a possible diagnosis for unexplained septicemia or abortion.

Listeriosis has been occasionally reported as cause of illness in horses and in animals kept in zoological parks or other preserves. The organism was isolated from an equine corneal lesion (Sanchez et al. 2001) and an adult alpaca with concurrent *L. monocytogenes* septicemia and *Clostridium perfringens* infection (Seehusen et al. 2008). Reports among animals at zoological parks include perinatal septicemia in a stillborn Celebese ape (McClure and Strozier 1995), fatal disseminated listeriosis in bushy-tailed jirds (Tappe et al. 1984), and meningoencephalitis in an adult cougar with progressive neurologic disease leading to recumbency and death (Langohr et al. 2006). Twenty percent of wild-caught monkeys may have *L. monocytogenes* in feces or intestinal contents (Yoshida et al. 2000); listeriosis has been reported among nonhuman primates, with symptoms similar to those in humans (reviewed in Wesley 2007). Cynomolgus monkeys are resistant to experimental oral challenge (Farber et al. 1991), but rhesus monkeys have been used for dose-response modeling of perinatal listeriosis (Smith et al. 2008).

Listeria monocytogenes occasionally infects fish and was recovered from the brain, spleen, kidneys, and fillets of farm-raised channel catfish (Wang et al. 1998). Farmed trout and whitefish in Finland were also culture positive, with greater prevalence after rainy periods with increased runoff from local streams (Miettinen and Wirtanen 2006). Prevalence of listeriosis among salt-water fish is unknown.

Avian listeriosis has also been occasionally reported. Commercial broiler chickens developed encephalitis after exposure to *Listeria* from an unknown source (Cooper 1989). Fatal listeriosis occurs in pet birds (canaries and a cockatiel) (Shivaprasad et al. 2007; Akanbi et al. 2008). Broiler chicks were colonized with *L. monocytogenes* after oral challenge (Bailey et al. 1990), and colonization was reduced by prior administration of a competitive exclusion culture of normal cecal bacteria (Hume et al. 1998). Day-old turkey poults are susceptible to oculonasal or oral challenge (Huff et al. 2008). Poults stressed by cold developed synovial and liver infection following oral and aerosol challenge (Dutta et al. 2008). Turkey-processing plants are reservoirs for *L. monocytogenes*, with many strains of the environmentally predominant

serotypes (1/2a and 1/2b) harboring resistance to cadmium and the quaternary ammonium disinfectant benzalkonium chloride, as well as resistance to listeriaphages isolated from the environmental samples (Mullapudi et al. 2008; Kim et al. 2008). EC strains (ECI and ECII) are detected among the serotype 4b isolates from these plants (Eifert et al. 2005). As noted, sources of the bacteria colonizing these plants are not known. Birds may serve as occasional sources, but other sources (e.g., environment, human traffic, equipment) cannot be excluded.

Listeriosis in Humans

L. monocytogenes is an environmental organism with pronounced ability to contaminate food-processing environments, resulting in adulteration of processed, RTE foods. Zoonotic transmission from animals to humans is rare and manifests primarily as cutaneous abscesses (e.g., of veterinarians or farmers tending to delivery of calves with listeriosis). The pathogen can also be transmitted directly through contaminated milk and especially through soft, fresh cheeses made with raw milk (Swaminathan and Gerner-Smidt 2008). The coleslaw-associated outbreak of the Maritime Provinces in 1981 was attributed to cabbage contaminated in the field by sheep manure from a flock with listeriosis, although bacteria were not detected in the manure (Schlech et al. 1983).

As mentioned earlier, ruminants (especially cows) are a reservoir. The organism is frequently detected on cull cow hides and carcasses, particularly in cooler weather (Guerini et al. 2007). Dairy farm and dairy plant surveys indicate that *L. monocytogenes* contamination of in-line milk filters and bulk tanks is common (Latorre et al. 2009). Raw milk can contain *L. monocytogenes*, and it can also be isolated from the dairy plant environment (Kells and Gilmour 2004; Jayarao et al. 2006). In 2007, an outbreak in Massachusetts was attributed to environmental contamination of milk postpasteurization (Voetsch et al. 2007).

Human clinical listeriosis occurs most commonly in pregnant women, often resulting in abortion, and as septicemia and meningitis in immunodeficient adults (Farber and Peterkin 1991; Schuchat et al. 1991). The largest of several outbreaks of human listeriosis in the United States occurred in the Los Angeles county, as a result of ingestion of contaminated Mexican soft cheese made with unpasteurized milk. Ninety-three of the 142 cases were in pregnant women and their fetuses, with a case fatality rate of 63% for neonatal or fetal infections and 37% for nonneonatal infections (Farber and Peterkin 1991; Schuchat et al. 1991). As in other outbreaks, most of the nonpregnant patients suffered from some underlying immunodeficiency. Other prominent outbreaks in North America have included U.S. multistate outbreaks associated with hot dogs and deli meats in 1998–1999, 2000, and 2002 (Olsen et al. 2005; Gottlieb et al. 2006; Swaminathan and Gerner-Smidt 2008). A Canadian outbreak in 2008 involving contaminated RTE lunch meats resulted in >40 confirmed cases and ≥ 15 deaths (Wilson and Keelan 2008). U.S. outbreaks incriminating deli-style turkey meat prompted changes in product formulation (e.g., addition of lactate, diacetate, or nitrite to inhibit growth of *L. monocytogenes*).

In addition to outbreaks of listeriosis with typical severe outcomes (stillbirths, meningitis, septicemia), outbreaks of febrile gastroenteritis have also occurred. Unlike invasive listeriosis, such outbreaks have frequently involved nonpregnant adults without obvious underlying immunosuppression, and the implicated food has been contaminated at high levels (Kathariou 2002). A large febrile gastroenteritis outbreak in Italy (292 persons hospitalized) was attributed to highly contaminated corn salad distributed to school cafeterias for student lunches (Aureli et al. 2000). An intriguing recent report concerned an elderly patient with *Listeria*-associated febrile gastroenteritis who developed invasive listeriosis 2 weeks subsequent to recovery from gastroenteritis (Pichler et al. 2009), suggesting the possibility that the latter may contribute to the disease burden associated with systemic listeriosis. Febrile gastroenteritis cases tend to be underreported and, in most cases, lack bacteriological diagnosis, thus making it currently difficult to assess the extent of this contribution.

Temporary decreases in incidence of listeriosis have been noted in the United States, possibly in response to government advisories, product formulation changes, and regulatory surveillance (Tappero et al. 1995; Voetsch et al. 2007). However, incidence has not decreased consistently in the United States or Europe. Approximately 2500 cases and 500 deaths are attributed to listeriosis in the United States each year (Notermans et al. 1998; Hurd et al. 2000), for an annual incidence of 7.4 cases per 1 million population and a case fatality rate of 23% (Schuchat et al. 1992). Nonperinatal mortality

appears to have decreased substantially between 1990 and 2005 (Bennion et al. 2008). Increased risk for fatal outcomes has been associated with listeriosis that accompanies human immunodeficiency virus infection, lymphoid and hematopoietic cancers, and liver disease (Bennion et al. 2008). Listeriosis incidence in several European nations appears to be on the rise among the elderly, a population always at risk for foodborne listeriosis (Goulet 2008).

An expert panel identified certain categories of foods that pose especially high risks for human listeriosis. These foods are (***1***) RTE (i.e., consumed without further cooking or other processing that would kill the bacteria), (***2***) preserved under refrigeration for substantial lengths of time, or (***3***) permissive for growth of the bacteria (ILSI 2005). Foods processed in a plant constitute a higher risk since environmental bacteria may be transferred onto foods after treatments, such as pasteurization (Kathariou 2002). Foods most commonly implicated in human listeriosis include soft cheeses, delicatessen meat items, undercooked chicken and hot dogs, hummus, and processed seafood (FDA-USDA-FSIS-CDC 2003; Varma et al. 2007).

Identifying the original source of *L. monocytogenes* contamination in foodborne infections can be difficult. It is likely that contamination occurs most frequently at the food-processing plant or at a food preparation facility rather than on the farm (Farber and Peterkin 1991). Nonetheless, as noted, ruminants are a reservoir for *L. monocytogenes*. For this reason, and because listeriosis has achieved considerable visibility as a food safety concern among regulatory agencies and the public, veterinary practitioners and diagnosticians are expected to assist food animal producers in reducing the likelihood that the animals are infected with *L. monocytogenes* preharvest. This would reduce the pathogen load in processing plant environments and the likelihood that dairy products (e.g., milk and soft cheeses made from it) would be contaminated.

HOST–PATHOGEN INTERACTIONS IN LISTERIOSIS

Most of the available information on host defense against listeriosis is based on experimental studies of murine listeriosis. Few published reports address host defense against listeriosis in cattle or sheep despite the acknowledged role of *L. monocytogenes* as a pathogen of ruminants. Information presented here is based principally on murine studies, with relevant reports from cattle and sheep discussed where available.

Innate Immunity

Given the ubiquity of *L. monocytogenes* and the infrequent occurrence of listeriosis, it is reasonable to assume that innate immune mechanisms effectively control occasional exposure to low numbers of organisms. The first barrier may be posed by the low pH of the stomach and competing microbes in the GI tract. *L. monocytogenes* does not multiply to high numbers in the GI tracts unless experimental animals are inoculated with large numbers of organisms (Lecuit et al. 2001) or the host is treated with agents that facilitate infection via the GI tract (Czuprynski et al. 2003a, 2003b).

Host secretions may also contain components that inhibit *L. monocytogenes*. Bovine lactoferrin has been reported to reduce invasion into an intestinal epithelial cell line and to inactivate LLO *in vitro* (Moriishi et al. 1999; Valenti et al. 1999). Human lactoferrin inhibited growth of *L. monocytogenes in vitro* and had some beneficial effect on host defense when administered to experimentally infected mice. Lipids in bovine milk inhibited *L. monocytogenes in vitro* (Sprong et al. 2001) and *in vivo*, preventing intestinal colonization of rats (Sprong et al. 1999). However, the fat content of various milk products had no consistent effect on the severity of listeriosis when mice were inoculated intragastrically with bacteria in milk (Mytle et al. 2006).

Resistance to *L. monocytogenes* requires a cellular immune response. Antibodies are generated against extracellular antigens during infection, but cell-associated antigens elicit little or no antibody response (Bhunia 1997; Darji et al. 1998). These antibodies are not protective but may enhance ingestion of *L. monocytogenes* by phagocytic cells (Czuprynski et al. 1984). However, administration of an anti-LLO monoclonal antibody protects mice against infection (Edelson and Unanue 2000), presumably by decreasing the effectiveness of LLO in infected cells (Kohda et al. 2002).

The first wave of inflammatory cells to enter foci of infection usually comprises substantial numbers of neutrophils (polymorphonuclear leukocytes [PMN]). Formyl peptides produced by *L. monocytogenes* are potent chemoattractants and activating agents for murine PMN (Southgate et al. 2008), and

PMNs from several mammalian species (including cattle) kill the organism *in vitro* (Czuprynski et al. 1984, 1989). Mononuclear phagocytes also ingest *L. monocytogenes* and, depending on the extent of activation, can kill a proportion of them (Czuprynski et al. 1984 1989; Portnoy et al. 1989). Mice treated with a monoclonal antibody that prevents PMN influx or depletes mice of circulating neutrophils were exquisitely sensitive to listeriosis (Conlan and North 1991; Czuprynski et al. 1984). Mice that lacked the molecule B7-H4 exhibited an increased PMN response and were more resistant to experimental infection than their wild-type counterparts (Zhu et al. 2008). It has been proposed that PMNs protect the host by destroying infected cells that harbor the listeriae (Gregory and Wing 2002) and by ingesting and killing listeriae released from the lysed cells (Czuprynski et al. 1984), but it is also possible that PMNs release mediators (i.e., cytokines) that activate the anti-*Listeria* activity of hepatocytes and macrophages (Edelson and Unanue 2000; Gregory and Wing 2002; Yin and Ferguson 2009). PMN may also assist in cross presentation of *L. monocytogenes* antigens by dendritic cells and priming of CD8+ T cells (Tvinnereim et al. 2004).

Cells of the innate immune system possess receptors for pathogen-associated molecular patterns (PAMPs), such as peptidoglycan, lipopolysaccharide, lipoteichoic acid, and flagellin that are on the microbial surface. Families of PAMP receptors include the Toll-like and nucleotide-binding oligomerization domain (NOD) receptors. Optimal resistance to murine listeriosis is dependent on Toll-like receptor 2 (TLR2), which binds bacterial lipoproteins and peptidoglycan fragments (Torres et al. 2004). *L. monocytogenes* triggers a vigorous cytokine response in human embryonic kidney cells transfected with bovine TLR2 (Farhat et al. 2008), and TLR5 responds to *L. monocytogenes* flagellin (Hayashi et al. 2001). The adaptor protein MyD88, which is involved in signaling from Toll receptors, is essential for resistance to infection (Edelson and Unanue 2002). Several members of the NOD receptor family also influence the macrophage response to *L. monocytogenes* (Warren et al. 2008; Leber et al. 2008).

Adaptive Immunity

The pioneering studies of Mackaness and coworkers in the 1960s demonstrated the preeminent role of cellular immunity in host defense against *Listeria* infection (Miki and Mackaness 1964). For many years, investigators adhered to a simple paradigm in which IFN-γ released by T helper cells activated macrophages to kill intracellular listeriae. While macrophage activation occurs in listeriosis, the protective host response is considerably more complex. Considerable multiplication of *L. monocytogenes* occurs in hepatocytes and other nonprofessional phagocytic cells (Havel 1989; Conlan and North 1991; Gregory and Wing 2002), indicating that additional defense mechanisms must be operative to achieve the sterilizing immunity observed in experimentally infected mice. CD4+ and CD8+ cytolytic T cells can lyse *L. monocytogenes*-infected macrophages (Pamer 2004). CD4+CD25+ T regulatory cells that arise during murine infection suppress the resulting CD8+ memory T cell response (Heit et al. 2008). CD8+ T cells may attack other types of cells infected with *L. monocytogenes,* as in viral infections. More recent evidence suggests that TH17 cells may also participate in host defense against *L. monocytogenes* infection (Orgun et al. 2008). It is also likely that natural killer (NK) cells release IFN-γ early in infection and that this is of considerable importance to host defense (Dunn and North 1991). This is not to say that macrophages play only a minor role; IFN-γ-treated activated macrophages restrict escape of *L. monocytogenes* from the phagolysosome (Portnoy et al. 1989). Thus, macrophage activation reduces intracellular multiplication of listeriae and increases the ability of macrophages to inactivate ingested organisms.

There is also abundant literature on the influence of various soluble mediators (cytokines) on resistance. The manner and timing with which these arise and interact are complex and undoubtedly crucial to the outcome of infection (Pamer 2004). Older work showed that administration of recombinant cytokines (e.g., IFN-γ, IL-1, IL-2, TNF-α, GM-CSF) enhanced resistance to experimental listeriosis in mice (Czuprynski and Haak-Frendscho 1997; Edelson and Unanue 2000). Conversely, cytokine-neutralizing monoclonal antibodies and mice unable to produce cytokines and cytokine receptors have been used to elucidate the roles of endogenous cytokines in resistance to listeriosis. In particular, endogenous IFN-γ and TNF-α play critical roles. *In vivo* neutralization of either of these cytokines has dire consequences for the infected host (Havel 1989; Kaufmann 1993). Results of work with rodents and cats suggest that a Th1 cytokine response (characterized by IFN-γ and IL-2

production) protects and a Th2 response (characterized by IL-4 production) is detrimental to resistance (Edelson and Unanue 2000; Pedersen et al. 1998). Somewhat surprisingly, administration of type I IFN, an important molecule for innate defense against viral infection, resulted in impaired resistance to *L. monocytogenes* infection (O'Connell et al. 2004). More recent work shows that IL-17, produced by T-cell receptor (TCR) gamma delta cells, contributes to host defense during the early stages of *L. monocytogenes* infection (Hamada et al. 2008).

PREVENTION AND TREATMENT

Results of experimental work in rodents indicate that clearance of sublethal *L. monocytogenes* infection renders the host highly resistant to rechallenge (Miki and Mackaness 1964). These animals also display a strong delayed-type hypersensitivity response to listerial antigens, although this skin response could be dissociated from protective immunity (Mielke et al. 1988). This heightened, acquired cellular resistance is maximal for 2–4 weeks and then wanes over several months. During the period of maximal resistance, protection could be transferred to naive recipient mice via immune spleen cells or purified T cells but not with serum (Miki and Mackaness 1964). The level of resistance transferred in this way is substantial but less than that resulting from active immunization associated with infection. It is generally accepted that active immunization requires the administration of viable *L. monocytogenes* (Gudding et al. 1985; Vagsholm et al. 1991; Linde et al. 1995); killed organisms or bacterial proteins do not generate maximal protective cellular immunity. However, it is conceivable that development of new adjuvant or vaccine strategies might enhance the immunity elicited by subunit vaccines. The possibility that viable attenuated *L. monocytogenes* might be used as vector for conventional or DNA immunization against heterologous antigens was demonstrated in rodent studies (Mollenkopf et al. 2001).

Many outbreaks of listeriosis have been traced to poor quality silage that contained increased numbers of *L. monocytogenes.* Ensuring use of good quality, low pH silage, and other animal husbandry interventions will reduce incidence of listeriosis in sheep, goats, and cattle. As a result, there appears to be little economic justification for a listeriosis vaccination program in North America. In certain parts of Europe, however, vaccination with live attenuated strains of serotypes 1/2a, 1/2b, and 4b is a common practice that is reported to bring cost-effective reductions in the incidence of listeriosis in sheep (Vagsholm et al. 1991). There may be U.S. public health concerns associated with the use of live (albeit attenuated) strains of a pathogen for which there is zero tolerance in RTE foods. Furthermore, it is impossible to assess the extent to which such strains might be able to induce illness in immunocompromised humans (e.g., farm staff and animal handlers) or to persist in the environment. If the latter occurred, it might eventually contribute to food contamination and further complicate elimination of *L. monocytogenes* from food products.

Depending on the value and condition of the animal, antibiotic treatment of food animals with listeriosis may not be advisable. The cost of therapy and economic losses associated with withdrawal times for the antibiotics used must be taken into consideration. Mastitis has proven refractory to treatment in several instances. Latent udder infections might result in shedding of the organism in milk during transient immunosuppressive events, such as parturition (Wesley et al. 1989). Aborting dams may clear the infection without treatment. The current treatment of choice for human listeriosis is penicillin, in combination with gentamicin. Sulfamethoxazole-trimethroprim, erythromycin, and tetracyclines have also proven effective in human listeriosis, whereas cephalosporins are not recommended, even in combination with other antimicrobials (Farber and Peterkin 1991; Schuchat et al. 1991).

CONCLUSION

Investigation of the molecular pathogenesis and host response to *L. monocytogenes* in rodent and tissue culture models is in a dynamic state, with new contributions being made at a rapid rate. Unfortunately, the state of our understanding of the pathogenesis of listeriosis in ruminants has not kept pace. There is paucity of published information on bovine and ovine host defense mechanisms. Observations previously made in these species (e.g., centripetal invasion of *L. monocytogenes* along the trigeminal nerve) offer exciting possibilities as models for further study of the roles of *L. monocytogenes* virulence factors in CNS infection. A better characterization of the dose-response curve with *L. monocytogenes* infection via the GI tract, and of

the host–pathogen interactions that allow the organism to translocate across the intestinal mucosa and disseminate to target organs (fetus, CNS), would facilitate risk assessment of listeriosis in domestic animals and humans. This information in turn could contribute to eventual reductions in the incidence of listeriosis.

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11
Neurotoxigenic Clostridia

H. Böhnel and F. Gessler

INTRODUCTION

Botulism and tetanus have been known as diseases for many centuries. *Clostridium tetani* and *Clostridium botulinum* were classified as the etiologic agents during the time of Koch and Pasteur.

CLOSTRIDIUM TETANI AND *CLOSTRIDIUM BOTULINUM*

Neurotoxigenic clostridia, like other members of the genus, are gram-positive, anaerobic, rod-shaped, spore formers; older cultures may be gram variable. The rods are straight or pleomorphic and occur singly, in pairs, or in short chains. Longer filaments may occur in older cultures. Both are motile, and *C. botulinum* can be classified, in part, by its proteolytic and saccharolytic activities (Smith and Hobbs 1974; table 11.1).

Spores of *C. tetani* are spherical, causing the bacterium to take on the shape of a drumstick. *C. botulinum*, on the other hand, has central, oval, or subterminal spores that do not bulge the cell. Spores may germinate and form vegetative cells within 30 min under ideal conditions (fig. 11.1). Viable but nonculturable bacteria occur, and superdormant spores do not germinate under normal laboratory conditions. This is especially true for newly formed spores (Smith 1975).

The short generation time of *C. botulinum* may lead to rapidly fatal disease in chickens after inoculation by as few as ten type A spores (Miyazaki and Sakaguchi 1978). *C. tetani* and *C. botulinum* may germinate and multiply in anaerobic microenvironments within otherwise aerobic environments (Akulinicheva 1989). The optimal temperature for growth and toxinogenesis of *C. tetani* is ~37°C, but for the various types and strains of *C. botulinum*, it may range up to 45°C.

Neurotoxigenic clostridia are soil bacteria (Smith and Sugiyama 1988). They are isolated from virgin soil and widely distributed globally, and the original occurrence of certain strains was geospecific. However, *C. tetani* is a frequent inhabitant of the gastrointestinal (GI) tract and associated tissues of herbivores and omnivores (Umesaki et al. 1999). *C. botulinum* type C spores were found in livers of apparently healthy pigs at slaughter (Yamakawa et al. 1992), and 62% of porcine fecal samples in Sweden yielded isolates of type B strains (Dahlenborg et al. 2001). *C. botulinum* type B and types E and F were isolated from 73% and 5% of cattle, respectively (Dahlenborg et al. 2003). Thus, global transport of animals and animal products is likely leading to widespread distribution of types previously uncommon in certain regions (Boyer et al. 2001).

Clostridium botulinum may produce biofilms, and planktonic bacteria behave quite differently from their sedentary counterparts (Kreft 2004). Reduced antimicrobial susceptibility contributes to persistence of biofilm-based infections, and the protective mechanisms appear to be other than standard antibiotic resistance (Stewart 2002). This may explain why *C. botulinum* is isolated from the feces of infants treated with antimicrobials for several months (Arnon et al. 1977).

Clostridia other than *C. botulinum* produce botulinum neurotoxin (BoNT), including type E toxin

Table 11.1. *C. tetani* and Groups of *C. botulinum* According to (A) Physiology and (B) Virulence (B)

(A) Neurotoxigenic *Clostridium* group	Neurotoxin	Other toxins	Phenotypically related nonneurotoxigenic *Clostridium* spp
C. tetani	TeNT	Tetanolysin	*C. tetanomorphum*
C. botulinum rroup I (proteolytic)	BoNT/A, B, F		*C. sporogenes*
C. botulinum group II (nonproteolytic)			
C. botulinum group III		C2, C3	*C. novyi*
C. botulinum group IV (*C. argentinense*)			*C. subterminale*
C. baratii	BoNT/F		
C. butyricum	BoNT/E		

(B) Neurotoxin type	Mainly affected species
TeNT	Horses, cattle, sheep, dogs, cats, other animals, humans
BoNT/A	Chickens, humans
BoNT/B	Horses, cattle, humans
BoNT/C	Birds, cattle, dogs, mink, ferrets, foxes, horses, pigs
BoNT/D	Cattle
BoNT/E	Fish, birds, humans
BoNT/F	Humans
BoNT/G	Humans?

TeNT—tetanus neurotoxin.
BoNT—botulinum neurotoxin.

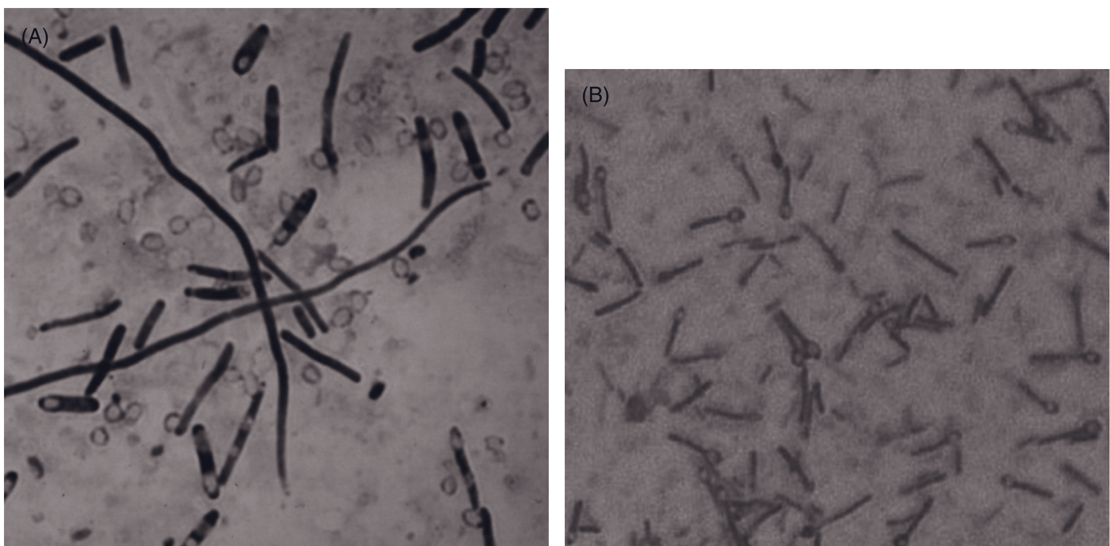

Figure 11.1. Microscopic view of bacterial forms and spores of (A) *C. botulinum* type C and (B) *C. tetani*.

by *Clostridium butyricum*, type F toxin by *Clostridium baratii*, and tetanus neurotoxin (TeNT)-like substances by *Clostridium cochlearum* (Sanada 1964). Nontoxigenic strains have lost virulence genes, and *C. botulinum* types C and D often undergo this change during isolation from pathological or environmental specimens (Smith and Sugiyama 1988; Johnson 2006).

TOXINS

The family of clostridial neurotoxins (CNTs) comprises some of the most potent toxins known. *C. tetani* produces TeNT, and *C. botulinum* and strains of *C. butyricum* (Fu and Wang 2008) and *C. baratii* (McCroskey and Hatheway 1984) produce the seven BoNTs (types A–G), including many toxin subtypes.

One might not expect structural and functional relationships between the toxins that cause spastic paralysis, on the one hand, and flaccid paralysis, on the other. However, these toxins are nearly identical in molecular mass and structure, and both inhibit release of neurotransmitters; the DNA sequence homology is ~65% (Lacy and Stevens 1999). CNTs are zinc metalloproteases synthesized as single chain proteins with molecular weights (MWs) of ~150 kDa. Posttranslational cleavage by bacterial or tissue proteases yields a dichain molecule linked by a disulfide bond. The C-terminal domain of the heavy chain (HC) (MW ~100 kDa) mediates binding to the presynaptic membrane of the neuronal cell, while an N-terminal domain is involved in translocation of the light chain (LC) from the endosome to the cytosol (Montecucco and Schiavo 1995). The LC (MW ~ 50 kDa) specifically cleaves proteins (SNAREs) that play key roles in the fusion of synaptic vesicles with the presynaptic membrane, thus inhibiting the neurotransmitter release.

TETANUS

Tetanus, derived from the Greek "tetanos" (contraction), was described in the fourth century BC in *Corpus hippocraticum* (Bizzini 1979). Wound contamination by soil (Nicolaier 1884) and purulent secretions (Carle and Rattone 1884) were suggested to lead to disease. Toxic substances were found in pure cultures of *C. tetani* more than 100 years ago, and we now know that TeNT is of a single antigenic type (Brandis et al. 1994).

Toxins

TeNT

The minimum lethal dose of TeNT, by the parenteral route, is ~0.1–3 ng per kg in humans and many other animals (Gill 1982). Horses are the most sensitive of domestic animals. TeNT lacks the protection from gastric acidity afforded BoNT by other proteins, possibly explaining its lack of toxicity via the oral route.

Contaminated puncture wounds may serve as a portal of entry for *C. tetani*. Growth of contaminating facultative organisms may create an anaerobic microenvironment, but another major factor is the ischemia often associated with wounds. Castration and dehorning, especially with elastrator rings, may provide suitable tissue environments for the multiplication of *C. tetani* and production of TeNT. However, superficial lacerations may also become infected. The umbilicus of newborn foals and other species (including humans) may be an entry point and site of toxinogenesis. In rare cases, toxinogenesis takes place in the GI tract.

TeNT secreted into tissue enters motor neurons, a process mediated by binding to ganglioside and sialic acid (Chen et al. 2008). TeNT undergoes retrograde transport (Erdmann et al. 1975) and reaches inhibitory interneurons in the spinal cord transsynaptically (Schwab et al. 1979). There, the LC cleaves vesicle-associated membrane protein (VAMP, also called synaptobrevin) (Schiavo et al. 1992), which is essential for fusion of synaptic vesicles with presynaptic membranes. Thus, release of neurotransmitters is blocked.

The structural gene for TeNT, *tent*, is on a large plasmid in *C. tetani* (Finn et al. 1984). It is preceded by *tetR*, and regulatory protein TetR in turn activates the *tent* expression (Marvaud et al. 1998). TetR is an alternative sigma factor that likely belongs to a subgroup of the σ^{70} family (Raffestin et al. 2005). Toxin production is ultimately regulated by nutritional and microenvironmental factors, but the molecular mechanisms are unknown.

Other Toxins in **Clostridium tetani**

Tetanolysin, a cholesterol-dependent cytolysin (Matsuda et al. 1993), belongs to a family of toxins found in clostridia and other gram-positive bacteria (Tweten 1997). Other potential virulence factors have been identified in the genome of *C. tetani* (Brüggemann and Gottschalk 2004).

Clinical Features

The incubation period for tetanus is 1–3 weeks, averaging 10–14 days. In rare cases, it could be much longer. Onset of symptoms in sheep may be seen 3–10 days after castration or docking. Infection of the umbilical stump may become evident as early as 48 h after birth.

Signs are similar across species. Initially, animals are reluctant to move, with rigid muscles, tremors, and stiffness in the neck and hindquarters. One to three days later, generalized muscular contractions occur, the head and neck are extended, the tail is held out, the limbs assume a sawhorse position, and muscles have a wood-like tension. Clapping of hands, sudden exposure to bright light, or even touching the animal may cause overreaction with increased tonic convulsions. Spasms last initially for seconds or minutes but later become continuous. Animals fall and are unable to rise. Feeding may become more difficult, there may be difficulties with mastication, swallowing is almost impossible, and salivation is increased. As disease progresses, the animals develop trismus ("lockjaw"), and the third eyelid of dogs and horses protrudes, with flaring of the nostrils and a wide-eyed expression. Facial spasms give rise to the classic "risus sardonicus" in horses and dogs. Sick animals perspire, and there is difficulty with respiration, urination, and defecation. Ephemeral spasms may be localized to one limb or body region near the site of injury in mild cases. The fatality rate is up to 80% in nonvaccinated animals.

Human tetanus is a severe polysystemic disease, affecting muscles and also autonomic nerves, endocrine and cardiovascular systems, lungs, kidneys, and the GI tract (Cook et al. 2001). TeNT blocks the inhibitory synapses, leading to spasms in overreacting muscles. Excitatory synapses may be attacked later, leading to flaccid paralysis in animals surviving early acute effects (Takano et al. 1981).

Diagnosis

Recognition of clinical symptoms is straightforward, but nonspecific cerebral symptoms seen in early tetanus may suggest rabies. TeNT is not detected by laboratory tests when the toxin is moving toward its target via the neural route. In some cases, TeNT may be found in the circulation, but tetanus is not known as a true toxemia (Johnson 2006).

Postmortem examination and laboratory confirmation are not usually necessary. However, when the diagnosis is unclear, samples should be collected and preserved before antimicrobial therapy. TeNT can be detected via a mouse bioassay, with and without neutralization with specific antisera. Wound samples are examined by bacteriologic culture (Akbulut et al. 2005).

Similar cerebral or CNS symptoms may be seen with intoxication by strychnine, especially in dogs. A protracted course of disease, accompanied by a history of use of this poison in the vicinity and accompanied by either normal or reduced body temperature, points toward strychnine poisoning. Borna disease and other viral encephalomyelitides may be confused with tetanus in horses, and bovine spongiform encephalitis (BSE) should be considered in cattle. Flaccid paralysis resembling botulism may be seen.

Treatment

When clinical signs have become obvious, the prognosis is poor, even with treatment, and most affected animals will die. Treatment in advanced stages is discouraged for ethical reasons. Before a final diagnosis of clostridial neurotoxicosis, supportive therapy may be initiated; if so, it should be continued for ≥4 weeks in mild cases.

Equine hyperimmune serum administered IV may neutralize free toxins in the circulatory system but has no effect on toxin molecules that have reached their target cells. Thus, the clinical picture may worsen, even after treatment. Injection of antitoxins directly into the subarachnoid space is preferred (Muylle et al. 1975). Antitoxins may cause allergic reactions, even in horses. Injection of toxoid with tetanus antitoxin has empirically recognized positive effects.

Nonvaccinated horses with wounds should be treated with both antimicrobials and tetanus antitoxin. Antimicrobials may not reach the damaged tissue via blood, so fresh wounds should be debrided and treated directly with penicillin or tetracycline.

Muscle relaxants or sedatives may counteract TeNT action. Animals should be kept in a dark and quiet environment, on soft bedding to avoid decubitus. The eyes should be protected against mechanical insult.

Prevention and Control

Veterinary vaccines consist of formol toxoid and adjuvants. A primary two-dose series with a 4–6-week interval and annual boosters are recommended

for all animals. Mares should receive a booster 4–6 weeks before foaling. This results in colostral immunity for 2–3 months (Ishii et al. 1989). Foals of vaccinated mares are vaccinated at 4–6 months of age, whereas foals of mothers with no history of vaccination should be immunized at 1–4 months of age. There is some sentiment, on the other hand, that foals of immune mares should not be vaccinated until at least 6 months of age due to the possible negative influence of maternal antibodies (Wilson et al. 2001).

BOTULISM

An exhaustive clinical description linked cases of botulism in southern Germany to consumption of certain types of sausage (Kerner 1820), and the term botulism was coined (Latin *botulus*, for sausage) for the condition often known then as Kerner's disease. An organism, named *Bacillus botulinus*, was isolated from the liver of a disease victim and the ham that he had consumed (Van Ermengem 1897).

Types of *C. botulinum*, based upon BoNT, were described more than 100 years ago. These were types A–G (also known as *Clostridium argentinense*; Giménez and Ciccarelli 1970). There may be as many as 100 mixed and mosaic types and subtypes, as well as strains with variations in the toxin cluster and strains with apparent geospecificity.

Toxins

BoNTs

Clostridium botulinum was isolated, and the toxic nature of culture supernatant fluid was described in the late 19th century (Van Ermengem 1897). Seven serologically distinct neurotoxins (BoNTs/A–G) that differ in structure, toxicity, and species specificity have been described (table 11.1). Most strains of *C. botulinum* produce one neurotoxin, but a few produce two [e.g., Ab, Af, Ba, or Bf (Giménez and Giménez 1993)]. Reports of dual production of types C and D toxins are spurious; polyclonal antibodies against either of these toxins label both due to the presence of common epitopes (Oguma et al. 1980; Hunter and Poxton 2001). Some strains of types C and D produce mosaic toxins, composed of parts of both molecules (Moriishi et al. 1996).

BoNT production is not limited to *C. botulinum*. *C. butyricum* can produce BoNT/E, causing infant botulism, and *C. baratii* producing BoNT/F has been isolated from infant and foodborne botulism (McCroskey et al. 1991).

Differences in antibody binding affinity suggested, and DNA sequencing confirmed, the existence of BoNT subtypes. BoNTs/A1–A4, B1–B3 and bivalent B, and E1–E6 have been reported (Smith et al. 2005, 2007; Hill et al. 2006; Chen et al. 2007) and may impact sensitivity of detection assays and the value of potential therapeutics. Evaluation of established methods to detect all known subtypes is a high priority, and current and novel therapeutics (e.g., antibodies) or prophylactic products (e.g., vaccines) must cover all subtypes of clinical relevance.

BoNTs associate with proteins to form complexes (BoNT complex [BNC]), also called progenitor toxins, which differ in size and structure depending on the toxin type, culture conditions, and microenvironment (Johnson and Bradshaw 2001). These complex proteins include a nontoxic, nonhemagglutinin (NTNH) component and several hemagglutinins (HAs). Other proteins and RNA may also be involved (Johnson 2005). Three BNCs of BoNT/A include the M complex, the smallest, that consists of the NTNH and BoNT (12S, MW ~ 300 kDa). The L complex (16S, MW ~ 500 kDa) includes several HAs, and the LL complex, (19S, MW ~ 900 kDa) is likely a dimer of the L complex (Sakaguchi et al. 1984). Other BoNTs important in animal botulism (e.g., C and D) appear in complex forms L and M (Oguma et al. 1999). BNCs are stable under acidic conditions, protecting the BoNT component through gastric acidity and dissociating from it at pH ≥ 7.2.

The potency of BoNT varies with toxin type and host species, ranging from 0.1 to 1 ng/kg if given parenterally (Gill 1982). Toxicity is up to 1000-fold less when types A and B are given orally (Johnson 2005).

Toxin crosses the GI barrier by endocytic and transcytotic processes, reaching the vascular system and lymphatics. HAs may be involved in binding to GI epithelial cells (Simpson 2004). BNCs reaching the vascular system dissociate and liberate BoNT. The blood-brain barrier is not permeable to BoNT (Simpson 2004), but it leaves the vasculature, by unknown mechanisms, en route to its target in the peripheral cholinergic nerve endings. BoNT binds to cell surface receptors at the presynaptic membrane, inducing endocytotic uptake. Synaptic vesicle protein SV2 and ganglioside are receptors for BoNT/A (Dong et al. 2006), as are the luminal domain of synaptotagmin II and

ganglioside for BoNT/B (Chai et al. 2006; Jin et al. 2006). These intriguing studies proved the dual-receptor concept proposed earlier (Montecucco et al. 2004). BoNT undergoes a pH-induced structural change in endosomes, leading to translocation of the LC from the luminal to the cytosolic surface of the endosomal membrane. Reduction in the disulfide bond releases LC into the cytosol, where it specifically cleaves proteins that mediate the fusion of neurotransmitter vesicles with the presynaptic membrane, blocking acetylcholine release. BoNT/A and E cleave synaptosome-associated protein (SNAP)-25, BoNT/C cleaves SNAP-25 and syntaxin, and BoNTs/B, D, F, and G cleave VAMP (Simpson 2004).

Genes encoding BoNT and the associated proteins are clustered. The genomic location of the cluster varies with type and subtype. In BoNTs/A, B, E, and F, the cluster is generally chromosomal. In BoNTs/C and D, it is bacteriophage related, possibly explaining the rapid loss in toxigenicity during isolation and storage of cultures. The gene cluster in BoNT/G and in several BoNT/A and B strains (e.g., BoNT/A3 Loch Maree, BoNT/B1, and bivalent B BoNT Ba4) are plasmid borne (Marshall et al. 2007; Smith et al. 2007). The position in the cluster of *botR*, which encodes a regulatory protein related to TetR in *C. tetani*, also varies with strain.

Toxin expression is likely to occur in late log and early stationary phases and is regulated by nutritional and microenvironmental factors (Bradshaw et al. 2004).

Other Toxins in Clostridium botulinum

Two additional toxins are produced by some strains of types C and D. C2 toxin belongs to a family of binary toxins with adenosine diphosphate (ADP)-ribosylating activity. It depolymerizes F actin, destroying the cytoskeleton (Aktories et al. 1986). The biological effects *in vivo* are increased vascular permeability (Ohishi et al. 1980), edema, and fluid accumulation in intestinal loops (Ohishi 1983; Ermert et al. 1997). It affects immune and smooth muscle cells (Aktories et al. 1992). C3 toxin ADP ribosylates guanosine triphosphate (GTP)-binding Rho proteins (Aktories et al. 1987), altering epithelial and endothelial barriers (Nusrat et al. 1995) and compromising the activities of immune cells (Millan and Ridley 2005; Vogelsgesang et al. 2007). The roles of both C2 and C3 toxins in pathogenesis of botulism require further investigation.

Botulism

Botulinum toxicoses often have devastating effects, with losses in herds of cattle and horses approaching 100% and 50%, respectively (H. Böhnel and F. Gessler, unpublished data). Spectacular outbreaks of botulism have been experienced since the last decades of the 20th century (Böhnel and Gessler 2003; Rocke and Friend 2008).

Disease may take two forms. Spores may be introduced into feed from soil during harvest or handling. Silage offers anaerobic niches for proliferation of *C. botulinum*, especially when there is incomplete acidification by lactic acid bacteria. Organic proteins and spores from carcasses of small animals chopped together with the forage increase the risk. BoNTs produced in this or similar environments are ingested or, in rare cases, inhaled. This is intoxication, the classical form of botulism.

Spores or vegetative bacteria introduced into the body via the GI tract, where the organism can multiply and produce BoNT, cause toxicoinfection (Meyer 1928). The evolutionary advantage of toxin production remains obscure.

Acute Disease

The intensity and rapidity of onset of clinical signs depend upon the BoNT type or subtype, the amount of toxin absorbed, and the duration of toxin action. The site of toxin absorption, the action of C2 and C3 in concert with BoNT/C and D, susceptibility of the intoxicated species (including its immune status), concomitant diseases and antimicrobial therapy, and feeding management are also important. Active animals are more often affected than inactive ones; they ingest more feed, and BoNT uptake by target cells is increased due to their higher rate of metabolism. Large amounts of BoNT may cause sudden death, whereas smaller amounts absorbed over a longer period may cause flaccid paralysis.

At the outset, animals have a stiff gait, tremble, and have difficulty rising and laying down; they often remain recumbent. Some animals continue feeding even when unable to rise. Popoff (1989) described "atypical" cases in cattle, with anorexia, dehydration, hypersalivation, and regurgitation of liquids but no limb paralysis.

Progressive paralysis of the esophagus and the tongue eventually makes feed uptake difficult (fig. 11.2); the animal will chew but not swallow. Feed may fall out of the nostrils or the mouth, and excess

Figure 11.2. Dairy cow with end-stage botulinum toxicosis.

Figure 11.3. Hen with botulism.

salivation may occur. Rumen hypotonia is common. Unlike rabies, there is no hydrophobia; failure to drink induces rapid dehydration. In areas with phosphorus deficiency (e.g., South Africa or South America), cattle develop pica, chewing the bones or carcass remnants of their comrades that died of botulism and ingesting the BoNT that killed them (Theiler and Robinson 1927). Sheep, goats, and wild ruminants may have signs similar to those in cattle (Radostits et al. 2000; Böhnel 2005).

Affected horses drag their hooves or stumble. They lack facial expression and appear sleepy while remaining alert. Nasal discharge, sweating, mydriasis, and irregular pupillar reflexes may be observed. Respiration is labored, and animals may collapse and die from 18 h to a few days after onset. Infection of equine gastric ulcers may lead to acute botulism. Foals with umbilical or GI infection ("shaker foal syndrome") usually die 24–72 h after onset of symptoms.

Dogs and cats seem less sensitive to BoNT, but there are a few reports of intoxication in the former (Smith 1975). There have been cases in captive tigers and lions (H. Böhnel and F. Gessler, unpublished data), and 52,000 foxes were reported to have died in a single episode in Finland (Lindström et al. 2004).

Recovery from mild disease usually ensues after several days, but the course may also be biphasic. A first attack, due to ingested toxin, leads to some degree of GI paralysis. Ingested spores may then produce toxin *in vivo* which, when absorbed, causes the secondary phase after 2–3 weeks.

Some strains producing BoNT/C or D also produce the toxic, but not neurotoxic, metabolites C2 and C3. C2 toxin increases permeability of blood vessels and causes lung hemorrhage and edema. Fever, coughing, and diarrhea may be the predominant symptoms in these cases. The mode of action of C3 is unknown. GI tract hemorrhages may signal the presence of this toxin. Outbreaks in modern cattle production systems normally cause devastating losses. Mortality may be as high as 95% (unpublished observations) and surviving animals may not return to normalcy. Humans with botulism may have residual signs as long as 5 years after recovery (Gottlieb et al. 2007).

The rate of botulism in poultry is increasing, as is the number of affected animals in individual outbreaks. The first symptom, limberneck (fig. 11.3), is normally followed by complete paralysis (Durant 1928). Death of waterfowl may be by drowning (Clark 1987). Insect larvae accumulate BoNT when feeding on carcasses of animals that died of botulism, and birds consuming these larvae develop botulism in turn (Böhnel 2002). Consumption of decaying plant material by water birds may lead to intoxication (Azuma and Itoh 1987; AFSSA 2002; Rocke and Friend 2008). The same environment may be associated with outbreaks of botulism in fish (Cann and Taylor 1982).

Chronic Disease

Clinical pictures not congruent with acute disease were encountered during investigation of several thousand cases of botulism in Germany and neighboring countries (Böhnel and Gessler 2003). Abnormal cases in which BoNT or *C. botulinum* were demonstrated were named "visceral botulism" (Theiler and Robinson 1927; Schwagerick and Böhnel 2001; Böhnel et al. 2001). Toxinogenesis in

the jejunum and colon is accompanied by continual absorption of small amounts of BoNT (Böhnel and Gessler 2005). The main symptoms are reduced milk production, indigestion (constipation alternating with diarrhea), frequent urination, dehydration or edema, lameness associated with noninfectious laminitis, abnormal stance, circulatory problems with engorged veins and venous pulsation, respiratory disorders, tucked-up abdomen and arched back, ruffled fur, weak or stillborn calves, wasting in heifers, infertility, and sudden death after short recumbence. Calf mortality (up to 2 weeks after birth) and abomasal displacement (Constable 2005) may be associated with this syndrome. Signs were reduced or eliminated by vaccination (H. Böhnel, unpublished data).

The human health risk associated with chronically infected animals and food originating from these animals is unknown but is considered negligible. Milk of animals with acute botulism does not contain BoNT (Pamukcu 1954; Moeller et al. 2003). BoNT and *C. botulinum* have been detected in udder tissue of cattle with botulism, but they were found in milk only if the udder was infected and there were measurable changes in milk quality (Böhnel et al. 2004; S. Reinmuth, unpublished data). The persistence of BoNT type E in fish has implications in public health.

Diagnosis

Treatment is likely to fail, and any degree of success depends upon early diagnosis. Concomitant medications may mask symptoms of botulism, and the full range of clinical signs may not be present and in equal intensity in every case.

Flaccid tail and drooping ears in cattle are indicative of acute toxicosis but not specifically intoxication as opposed to infection. Cattle in chronically infected herds are often quite silent and lethargic in their movements and have reduced milk production and feed conversion and increased calf mortality (Böhnel et al. 2001). Feeding practices and changes in feed components should be investigated.

Except for cases of classical intoxication, where paralysis of the legs and tongue is pathognomonic, suspect animals should be necropsied and examined for microscopic lesions; appropriate specimens should be subjected to toxicologic analysis. Chronic botulism is often best confirmed by "diagnostic vaccination." Disappearance of clinical signs after vaccination strengthens, but does not absolutely confirm, the diagnosis (B. Schwagerick, unpublished data).

Botulism is the diagnosis of first choice in cases of acute flaccid paralysis in animals with normal or subnormal body temperature. Toxic plants, mycotoxins, organophosphates, and snakebites should be ruled out. Milk fever and hypocalcaemia may confuse the diagnosis in periparturient cattle. Feeds deficient in minerals or inadequate in other ways may cause similar clinical signs in high-yielding dairy cows. In peracute cases in cattle, pasteurellosis, heartwater, babesiosis, and tick fever should be ruled out, as should Newcastle disease or Marek's disease in poultry. Rabies should be considered first in cases with hypersalivation and ataxia. Equine acute pasture myodystrophy may be mistaken for botulism (Gerber et al. 2006). Grass sickness (Hunter et al. 1999; McGorum et al. 2003; Böhnel et al. 2003; Saeed 2005) and downer cow syndrome (Kelch et al. 2000; Green et al. 2008) should be ruled out, although both may occur concurrently with botulinum intoxication.

Laboratory Tests

Several specimens should be examined to confirm a diagnosis of botulinum toxicosis (table 11.2). Laboratory detection of BoNT still relies mainly on the mouse bioassay, and methods are similar to those used in the detection of TeNT. Animals inoculated with eluted specimen, with and without

Table 11.2. Specimens for Laboratory Diagnosis of Botulism

Live animal	Dead animal	Environmental samples
Blood/serum	Blood/serum	Feed (silage, TMR)
Rumen contents	Rumen contents, ileum, colon, cecum, rectum	Soil
Feces	Liver	Water
Saliva	Spleen	Carrion
	Tonsils	

TMR—total mixed ration.

antitoxin, are observed for clinical signs over a period of 96h. Alternative methods have been developed, including traditional enzyme immunoassays, lateral flow assays, detection of SNARE cleavage products by fluorescence or mass spectrometry, and cell-culture-based tests. PCR, qPCR, and other molecular tools have been developed, but most have drawbacks, including difficulty with sample matrices, lack of sensitivity, or lack of toxin quantitation (Lindström and Korkeala 2006; Cai et al. 2007).

CONTROL AND PREVENTION

Treatment

As noted, treatment is unlikely to be effective after onset of clinical signs and is discouraged for ethical reasons in advanced stages. Supportive treatment may be started before the final diagnosis and may be required for at least 4–6 weeks.

As with tetanus, antitoxins do not alter clinical signs. Circulating toxins are neutralized, but those at the site of action are not affected. Nonetheless, empirical results in cattle reveal that IV dosing with botulinum antitoxin may be effective, even if mild clinical signs have been observed (H. Böhnel, unpublished data). A single treatment is recommended, as protection will last normally for about 3 weeks. Antitoxins may cause allergic reactions, even in horses.

Vaccination

Vaccination against botulism is indicated only in areas where the disease is prevalent. It may prevent shaker foal syndrome (Lewis et al. 1981). Products for immunization against BoNT/C and D are available for use in cattle in some countries, as are BoNT/C and D and BoNT/ B products for horses (Jansen et al. 1976). "Long-acting" formulations require a single injection every 2–3 years for animals in regions with high prevalence (Turner and Simpson 2006). Biennial booster vaccinations may be necessary where chronic systemic disease is a problem. A recombinant vaccine against BoNT/C may improve immunity and reduce postvaccinal reactions (Frey et al. 2007). Similar findings have been reported in humans (Smith and Rusnak 2007). Mucosal vaccination may yield responses that will more readily neutralize the effects of BoNT delivered by aerosol (Fujihashi et al. 2007).

Management

Probiotics (lactobacilli) or prebiotics may impact the intestinal microbiota in such a way as to abrogate chronic botulinum intoxication. Classical prevention strategies are based upon reduction in the number of spores/vegetative forms in the environment. It is also important to be circumspect in use of organic material contaminated with *C. botulinum* (e.g., poultry manure) as fertilizer. Silage with pH higher than 5.5 and apparent mold growth should not be fed. Carcasses should be removed from pastures or from litter in poultry production. Use of rodenticides may increase the risk of botulism if feedstuffs are contaminated by decomposing carcasses.

BONT AS THERAPEUTICS

More than 100 disorders of humans can be treated with BoNT. Practical use in veterinary medicine remains uncommon, confined mainly to treatment of prostate dysfunctions or blepharospasm in dogs.

CONCLUSIONS

Clostridial neurotoxicoses are disease of longstanding which, in spite of intense study and efforts at control, are still of great importance. In the immediate future, greatest impact on these syndromes can be achieved by study of epidemiology, especially of disease caused by types and subtypes of BoNT. Information gained in this area will contribute to efforts to sharpen prevention and control materials and strategies. Elucidation of the roles of C2 and C3 toxins may also have an important impact on our understanding of pathogenesis of intoxications and perhaps increase the efficacy of vaccines. Finally, efforts to assemble better diagnostic methods, focused on the specific actions of the toxins, will almost certainly make management, prevention, and control of the disease more feasible.

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12
Histotoxic Clostridia

J. Glenn Songer

INTRODUCTION

Clostridial myonecrosis in humans and domestic animals can be associated with numerous species, but most cases are caused by a small group of these organisms. The infecting organism may come from soil, but the source is often endogenous, usually the intestinal tract. Entry to tissue may be by trauma, but evidence suggests that spores in the gut translocate to normal tissue, remaining dormant for the life of the animal unless presented with conditions appropriate for germination and growth. Local multiplication, extensive local and systemic tissue damage, and rapid death follow in most cases, and the hallmark is enthusiastic toxinogenesis. Renewed interest in mechanisms of pathogenesis has yielded new information, particularly about modes of toxin action.

VIRULENCE FACTORS AND PATHOGENESIS

The virulence factors that are known or suspected to be involved in disease and pathogenesis are discussed for each of four species of histotoxic clostridia.

CLOSTRIDIUM PERFRINGENS

Clostridium perfringens is the anaerobe most commonly found in human infections and the most important cause of clostridial disease in domestic animals (table 12.1). The species is divided into five toxinogenic types (Hatheway 1990), and although typing of myonecrosis strains is apparently rare, the assumption is that most are type A.

A common manifestation of *C. perfringens* infection is myonecrosis or fasciitis (Finsterer and Hess 2007). Rapid postmortem dissemination of *C. perfringens* from the gut often makes it difficult to assign meaning to its isolation from uncommon lesions. However, findings in humans suggest that the organism may produce fulminant and fatal central nervous system disease, including meningitis, encephalitis, cerebral abscess, or subdural empyema (Finsterer and Hess 2007). Lung infections are not uncommon (Brady et al. 2005). Most infections are traumatic, and experience suggests the occurrence of iatrogenic cases (J. G. Songer, unpublished). Infections can also originate in gastrointestinal or urogenital problems, or may be associated with malignancy (Finsterer and Hess 2007). Endomyometritis (“pink lady syndrome”) can follow cesarean delivery or bungled obstetrical procedures in human beings; early recognition and aggressive management may prevent fulminant and ultimately fatal disease (Cohen et al. 2007).

Regardless of the site of origin or the means of infection, dogma holds that spores in ischemic tissue germinate, vegetative cells multiply, and the infection spreads to healthy muscle. Toxins are the major virulence factors (Bryant et al. 2000; Awad et al. 2001). Alpha-toxin (CPA) is a Zn^{2+} metalloenzyme with lecithinase and sphingomyelinase activities. It comprises an N-terminal domain containing the active site and a C-terminal domain required for Ca^{2+} dependent interaction with membranes (Flores-Díaz and Alape-Girón 2003). Its several biological activities include increasing capillary permeability, induction of platelet aggregation,

Table 12.1. Clostridia as Agents of Myonecrosis

Clostridial species	Major toxins	Diseases	Species affected
perfringens type A	Alpha, theta	Gas gangrene, myonecrosis	All warm-blooded
septicum	Alpha	Abomasitis, malignant edema	Sheep, cattle
chauvoei	Alpha, beta	Blackleg	Sheep, cattle
novyi types A and B	Alpha, beta	Wound infections ("bighead"), infectious necrotic hepatitis	Sheep, goats
novyi type D	Beta	Bacillary hemoglobinuria	Cattle
sordellii	Lethal, hemorrhagic	Myonecrosis	Sheep, cattle

hemolysis, myonecrosis, decreasing cardiac contractility, and lethality (Flores-Díaz and Alape-Girón 2003). It may play both local and systemic roles in clostridial myonecrosis, the latter possibly by effects on platelets and phagocytes. Virulence of allelic exchange mutants of *pfoA* (perfringolysin O) and *cpa* revealed synergistic effects, in that infection with the double mutant resulted in minimal pathology. When complemented with both genes, the double mutant produced lesions more severe than when complementation was with *cpa* alone, supporting the hypothesis that synergy between CPA and PFO is important in the pathology of gas gangrene (Awad et al. 2001). Both toxins also influence occurrence of the characteristic vascular leukostasis (Ellemor et al. 1999).

A precise role for CPA is less clear. Myotoxicity depends upon membrane effects and alterations in blood flow. Dysregulation of transduction pathways in endothelial cells, platelets, and neutrophils (PMN) leads to uncontrolled production of intercellular mediators and adhesion molecules; diacylglycerol production promotes adhesion of PMN to fibrinogen and fibronectin (Ochi et al. 2002), resulting in altered movement of PMN to sites of infection and initiation of intravascular coagulation (Flores-Díaz and Alape-Girón 2003). The ischemia that follows promotes growth of *C. perfringens* (Flores-Díaz and Alape-Girón 2003).

Specific immunization of domestic animals against infection by *C. perfringens* type A is not practiced in North America, due to lack of availability of an appropriate vaccine and to the relatively uncommon and sporadic nature of the infection, but vaccines are available elsewhere in the world. The protective effect of CPA-containing toxoids against gas gangrene has been known for decades. Antibodies against native CPA and against a genetically truncated C-terminal portion of the molecule (amino acids 247–370) protect mice against challenge with toxin or multiple 50% mouse lethal doses (LD_{50}) of *C. perfringens* (Williamson and Titball 1993).

CLOSTRIDIUM SEPTICUM

Clostridium septicum is commonly found in soil and in the feces of domestic animals (Princewill and Oakley 1976). It is a frequent postmortem invader from the gut of domestic animals, particularly ruminants, and may enter the liver in one of the life stages of flukes (Gariev 1986). Iatrogenic infections (Harwood 1984) are more common in horses than in other species, and umbilical infections occur in sheep. "Braxy" in sheep or cattle follows invasion by *C. septicum* of abomasal mucosa damaged by frozen or poor-quality feed (Schamber et al. 1986). Hemorrhagic necrosis and edema are found in the abomasum and proximal small intestine (Ellis et al. 1983), and toxemia is common.

The organism is best known as the cause of malignant edema in domestic animals and humans, which is often associated with traumatic wounds, occult bowel carcinomas, diabetes mellitus, liver cirrhosis, and peripheral vascular disease (Chen et al. 2001). Nontraumatic clostridial myonecrosis is an uncommon but often fatal condition, with rapid onset, tachycardia, gas accumulation in soft tissues, and bullus formation (Burke and Opeskin 1999). Hemorrhage, edema, and necrosis develop rapidly as the infection spreads along muscular fascial planes. Pain and pitting edema at sites of lesion development give way to crepitance and decreased temperature, suggesting loss of circulation (Hickey et al. 2008), and the clinical course is often <24 h. The source of the organism is intestinal in more

than 50% of human patients and the mortality rate varies from 33% to 58% (Rechner et al. 2001).

Toxic or potentially toxic products of *C. septicum* include beta-toxin (DNase, leukocidin), gamma-toxin (hyaluronidase; Princewill and Oakley 1976), delta-toxin (oxygen-labile hemolysin), and neuraminidase (Gadalla and Collee 1968; Zenz et al. 1993), but the primary virulence attribute is alpha-toxin (Tweten 2001). Alpha-toxin is secreted as a 46,450 Da protoxin, which has a 72% amino acid sequence similarity to the primary structure of aerolysin from *Aeromonas hydrophila* (Ballard et al. 1995). Toxin specific activity is ~1.5×10^6 hemolytic units per mg, and the mouse LD_{50} is ≈10 µg/kg (Ballard et al. 1993).

Alpha-toxin apparently binds to glycosylphosphatidyl-inositol-anchored protein receptors (Gordon et al. 1999). It is activated by proteolytic cleavage of a C-terminal 45 amino acid fragment, a propeptide that may be an intramolecular chaperone; it stabilizes alpha-toxin monomers and escorts them to the membrane, where protease activation and oligomerization are followed by pore formation in the plasma membrane (Ballard et al. 1993; Sellman and Tweten 1997). Deletions in the transmembrane domain of alpha-toxin eliminate pore formation and any contribution of the toxin to pathogenesis (Kennedy et al. 2009). Furin and other eukaryotic proteases activate toxin on the cell surface *in vivo* (Gordon et al. 1997).

The brief clinical course dictates a focus on prevention rather than treatment, and death following *C. septicum* challenge is delayed in immunized mice. Antibody responses to toxoid yield lifelong immunity to malignant edema in domestic animals (Green et al. 1987; Hjerpe 1990), although differences in immunogenicity by host species have been reported (Green et al. 1987). In feedlot cattle, death losses were reduced by ~50% in vaccinates, with estimated savings of >$10 per animal due to vaccination twice at an average cost of less than $0.25 per dose (Knott et al. 1985).

CLOSTRIDIUM CHAUVOEI

Clostridium chauvoei causes blackleg, an emphysematous, necrotizing myositis (table 12.1), which resembles malignant edema (Burke and Opeskin 1999). Infected animals develop high fever, anorexia, depression, and lameness, with crepitant lesions and sudden death. Central areas of lesions are often dry and emphysematous, but peripheral areas are edematous, hemorrhagic, and necrotic, and leukocytic infiltration is negligible.

The roles of *C. chauvoei* alpha-toxin and beta-toxin (DNase) are undefined (Ramachandran 1969). Flagella expression is associated with virulence and subject to phase variation (Tamura et al. 1995); anti-flagella immunity is apparently protective (Kojima et al. 2000); anti-idiotypic flagella antibodies immunize mice against challenge (Kijima-Tanaka et al. 1994). Recombinant flagellin protein does not induce protection of mice against challenge (Kojima et al. 2000). Equine hyperimmune serum and penicillin can be used for therapy and prophylaxis.

CLOSTRIDIUM NOVYI

Clostridium novyi type A causes gas gangrene in humans and wound infections in animals. The hallmark lesion is edema, as in "bighead" of young rams; rapidly spreading edema of the head, neck, and cranial thorax follows invasion of subcutaneous tissues damaged by fighting. Infectious necrotic hepatitis ("black disease") of sheep and cattle results from *C. novyi* type B infection. Dormant spores germinate in liver tissue damaged by fluke migration, and systemic effects with acute or peracute death follow dissemination of alpha toxin (Elder and Miles 1957). Its cardiotoxic, histotoxic, and hepatotoxic effects produce edema, serosal effusion, and focal hepatic necrosis (Elder and Miles 1957). The name "black disease" derives from the characteristic darkening of the underside of the skin due to venous congestion. An emerging problem is apparently sudden death in sows, associated with multiplication of *C. novyi* type B in the liver at or about the time of parturition (Friendship and Bilkei 2006).

Clostridium novyi type D, often referred to as *C. haemolyticum*, causes bacillary hemoglobinuria (redwater) of cattle and other ruminants. Pathogenesis bears similarity to that of black disease, in that migration of immature flukes causes liver damage and predisposes to disease.

In addition to gas gangrene, *C. novyi* type A has been recently recognized with alarming frequency, as has *Clostridium botulinum*, as a cause of septicemia in drug addicts who had injected themselves intramuscularly (McGuigan et al. 2002; Ryan et al. 2001). Among 60 cases, the median age was 30 years, and most injected acidified heroin extravascularly. The case fatality was nearly 90% (McGuigan et al. 2002).

Differential production of alpha and beta-toxins is the major phenotypic differentiating factor. *C. novyi* alpha toxin belongs to the family of large clostridial cytotoxins that modify small GTP-binding proteins, affecting the cytoskeleton (Just and Gerhard 2004; Schirmer and Aktories 2004; Ziegler et al. 2008). Full enzyme activity of the intact toxin resides on a ≈550 amino acid fragment, and mutation of aspartic acid residues within this fragment dramatically reduces enzyme activity (Busch et al. 2000). Alpha-toxin specifically modifies the Rho subfamily proteins (Rho, Rac, Cdc42, and RhoG) by N-acetyl-glucosaminylation of Thr-37 (Selzer et al. 1996).

The beta-toxin of *C. novyi* type D is a phospholipase C (PLC; Hauer et al. 2004). It is a protein of 42,869 Da and exhibits a high degree of homology with other clostridial PLCs. Its N-terminus contains Zn^{2+}-binding residues common to these enzymes, and the C-terminus is highly homologous to the equivalent regions of *C. novyi* type A PLC, *C. perfringens* alpha-toxin, and *Clostridium bifermentans* PLC (Hauer et al. 2004).

Spores of genetically engineered nontoxigenic mutants of *C. novyi* administered intravenously germinate and produce tissue damage only under the reduced-oxygen conditions found in solid tumors. This approach is now in clinical trials (Wei et al. 2008).

There is no effective treatment for *C. novyi* infections, but prophylaxis with toxoids is generally effective. Second-generation veterinary vaccines may be based upon native or recombinant alpha toxoids (Amimoto et al. 1998).

CLOSTRIDIUM SORDELLII

Clostridium sordellii is an important pathogen of both humans and domestic animals. Deaths in human postsurgical patients almost exclusively followed knee replacement. This organism can also cause endometritis (postpartum or spontaneous), with sudden onset, flu-like symptoms, progressive refractory hypotension, and rapidly spreading edema. Laboratory findings are typically marked leukocytosis and elevated hematocrit. The clinical course is short, leading to a fatal outcome in most cases (Rorbye et al. 2000). More recently, *C. sordellii* has been implicated in endometritis and septic shock in humans after intravaginal use of the birth control drugs misoprostol or mifepristone (Aronoff et al. 2008). *C. sordellii* is also a common cause of necrotizing fasciitis in those injecting black tar heroin (Dunbar and Harruff 2007).

Clostridium sordellii was a potentially important finding in intestines of cattle experiencing "sudden death syndrome" (Manteca et al. 2001), and bovine enteritis has been experimentally reproduced with this organism (Al-Mashat and Taylor 1983). *C. sordellii* infection should be part of the differential diagnosis of the acute peritonitis associated with infection of internal umbilical remnants (Ortega et al. 2007).

Lethal and hemorrhagic toxins are antigenically and functionally similar to *Clostridium difficile* toxins B and A, respectively (Geny et al. 2007). Lung and heart are targeted preferentially in mice inoculated intraperitoneally with lethal toxin, and damage to endothelial cells caused increased vascular permeability. Lethal toxin glucosylates Ras, Rac, and Ral, differing from other large clostridial toxins in its modification of Ras. The role of a *C. sordellii* PLC in pathogenesis is not known (Karasawa et al. 2003). It has greater homology to *C. bifermentans* PLC than to alpha-toxin of *C. perfringens*, and its hemolytic activity was <1% that of CPA.

Immunization of guinea pigs with toxoids of lethal and hemorrhagic toxins protects against spore challenge, and both must apparently be in the vaccine (Amimoto et al. 2001).

CONCLUSION

Knowledge of the modes of action of toxins in clostridial myonecrosis has advanced substantially over the past decade. Structure and function of many of the toxins produced by histotoxic clostridia are well-defined if not completely understood. Our grasp of the biology of most, if not all, of the these organisms has improved significantly. However, the most notable improvements have been in genetic manipulation of clostridia, and production of mutants with which to study aspects of biology and pathogenesis, while not routine, is achievable in many instances. A further source of information will continue to be genomics. Mining data in genomic sequences has already led to deeper understanding of virulence attributes, and comparative genomics will increase our knowledge of virulence evolution.

The remaining question is when and how these new methods and information will be applied to solving animal health problems. It is now possible to prepare multivalent, highly-specific recombinant vaccines against many of these agents, for use in

many species. So-called "site reactions" and other undesirable post-vaccination effects could be greatly reduced or eliminated. These should become goals of the livestock and veterinary biologics industries.

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13
Enteric Clostridia

J. Glenn Songer

INTRODUCTION

Clostridia appeared early in the evolution of life, and, in fact, a gram-positive, eubacterial root has been suggested for the tree of life (Skophammer et al. 2007). Clostridia have evolved from that primitive ancestor, and representatives of the group were available to begin the evolution of parasitic relationships when the host itself appeared. Clostridia have an impressive array of virulence attributes and infect numerous systems in myriad hosts, remaining as common presentations to veterinary diagnostic laboratories. *Clostridium spiroforme* occurs commonly in rabbits but has also been associated with foal enteritis (J. G. Songer, unpublished), and *Clostridium colinum* is encountered consistently in poultry ulcerative enteritis, particularly in quail. However, enteric infections by the various toxinotypes of *Clostridium perfringens* are most important in their economic impact and overall incidence. *Clostridium difficile* has emerged as an important pathogen of horses, pigs, and other species, and *Clostridium septicum* enteric infections match its importance in histotoxic infections.

CLOSTRIDIUM PERFRINGENS

Clostridium perfringens is a common inhabitant of the intestinal tract of warm-blooded animals, but some toxinotypes are consistently associated with disease (table 13.1). It is an anaerobic, spore-forming, large gram-positive rod (Songer 1996), motile by way of type IV fimbriae (Varga et al. 2006). The organism produces ~20 toxic proteins, relatively few of which have a defined role in pathogenesis. Production of the so-called major toxins (alpha-toxin [CPA], beta [CPB], epsilon [ETX], and iota [ITX]) divides the species into five toxinotypes (table 13.1). All isolates from warm-blooded animals produce alpha-toxin (CPA).

There have been informal suggestions that the typing system has lost relevance in the molecular era and in light of the finding of multiple pathotypes within type A. Thus, there may be good reason to consider modernization of the typing scheme, but any move to do so should be tempered by attention to the familiarity and adequacy of much of the *status quo*.

Clostridium perfringens is an extraordinarily versatile pathogen, especially in the gastrointestinal tract. Judging by the rapidity of onset of some enteric infections caused by this organism, it is likely that transmission is from the dam in the form of vegetative cells. Redundant acid-resistance systems (see below) likely contribute to its survival during gastric transit, and the specific impact of various strains on the small intestine is determined by the type of genetic machinery it possesses. Data are consistent with a working hypothesis that acquisition of a specific virulence attribute (e.g., *cpb*, the gene encoding beta-toxin) has allowed the organism to occupy and evolve within a specific niche (the gut that has been protein starved or contains protease inhibitors; J. G. Songer, unpublished). Recent findings (e.g., description of CPB2, NetB, and TpeL) suggest that the organism continues the evolutionary process to better achieve its end goal of unfettered reproduction.

Complete genome sequences have been obtained for strains 13 (3.03 Mb; Shimizu et al. 2002), ATCC 13124 (3.26 Mb), and SM101 (2.9 Mb; Myers et al.

Table 13.1. Diseases Produced by Toxigenic Types of *C. perfringens*

Toxin type	Major diseases	Major toxins
A	Food poisoning, poultry necrotic enteritis, lamb enterotoxemia, porcine neonatal necrotizing enterocolitis, bovine neonatal hemorrhagic enteritis	Alpha
B	Lamb dysentery, ovine chronic enteritis, bovine/equine hemorrhagic enteritis	Alpha, beta, epsilon
C	Fowl necrotic enteritis, hemorrhagic or necrotic enterotoxemia in piglets, lambs, calves, goats, foals, acute enterotoxemia ("struck") in adult sheep	Alpha, beta
D	Ovine enterotoxemia, caprine enterocolitis, bovine enterotoxemia (calves, possibly adults)	Alpha, epsilon
E	Bovine (possibly ovine) neonatal enterotoxemia	Alpha, iota

2006). Genomic analysis revealed that virulence-associated genes do not form pathogenicity islands, and the G + C content of these genes suggests that extensive horizontal transfer from other bacteria is unlikely to have occurred or at least has not occurred recently. There are few insertion elements in strains 13 and ATCC 13124, but 69 in strain SM101 (Myers et al. 2006). Outside a conserved core, >300 unique "genomic islands" contribute to considerable genomic diversity. There are genes for enzymes enabling anaerobic fermentation but not for the TCA cycle or respiratory chain (Shimizu et al. 2002; Myers et al. 2006). Genes encode saccharolytic enzymes, but many enzymes for amino acid biosynthesis are lacking. This is congruent with the organism's production of degradative enzymes and toxins, by which it obtains essential nutrients from the host. The existence of multiple systems for acid resistance, including arginine deaminase, glutamate decarboxylase, and citrate fermentation pathways, may relate to the need for vegetative cells to survive gastric transit (Myers et al. 2006). Known virulence genes were identified, but potentially-virulence-associated genes included putative hemolysins, *Bacillus cereus* enterotoxin homologs, and putative fibronectin-binding protein and proteinase genes. One of the latter was a homolog of the *Clostridium histolyticum* α-clostripain (cysteine proteinase) gene. Strain 13 plasmid pCP13 (54 kbp) contains putative virulence genes for beta2-toxin and for a collagen adhesin.

Considerable effort has been invested in understanding gene regulation in *C. perfringens*, addressing both virulence and housekeeping genes. The two-component VirR/VirS system (VirR response regulator and VirS sensor protein), which shares features with other bacterial two-component systems, has received the most attention. A sensor histidine kinase and a response regulator, linked by a phosphorelay, enable responses to environmental prompts. Screening the genomic sequence for the VirR-binding consensus sequence allowed identification of genes potentially regulated by the VirR/VirS system, and this regulation has been confirmed unequivocally for perfringolysin O, collagenase, alpha-toxin, sialidase, protease, and hemagglutinin (Lyristis et al. 1994; Shimizu et al. 1994).

Diseases Caused by *Clotridium perfringens* Type A

Poultry Necrotic Enteritis

The worldwide economic impact of necrotic enteritis (NE) is $2 billion annually (Porter 1998). The peracute and acute forms of NE may be the most clinically dramatic bacterial infections of poultry (Porter 1998). Introduction of highly effective anticoccidial vaccines, decreased use of ionophore coccidiostatic drugs, and withdrawal of antimicrobial growth promotants have been accompanied by increased prevalence of NE.

Etiology. Necrotic enteritisis is caused mainly by *C. perfringens* type A, with occasional cases due to type C infection (Long et al. 1974). It is now known that specific strains of type A produce NE. Chicken

normal flora strains or strains from enteric disease in other species are not virulent for chickens in an experimental model of infection (Chalmers et al. 2007; Barbara et al. 2008; Chalmers et al. 2008; Timbermont et al.2008; K. K. Cooper and J. G. Songer, unpublished). However, even NE strains fail to establish and produce disease if intestinal conditions are not favorable. Linkage disequilibrium among isolates from Canadian NE outbreaks suggests a clonal population structure (Chalmers et al. 2008).

Signs and Lesions. Birds that are depressed, reluctant to move, and diarrheic (Porter 1998) may be at the beginning of a short clinical course culminating in acute or peracute death (Long et al. 1974). Mortality rates can be as high as 50% (Shane et al. 1985; Craven et al. 1999). Gross lesions are usually found only in the jejunum and ileum but can occur in the duodenum and ceca (Long et al. 1974; Porter 1998). The small intestine is usually distended with gas (Porter 1998), and the wall is thin and friable (Kaldhusdal and Hofshagen 1992). Villous necrosis is extensive (Long et al. 1974; Broussard et al. 1986) and may extend to the submucosa or muscularis mucosa. Coagulation necrosis occurs at villous apices, with accumulation of mononuclear cells between affected and normal tissues (Long et al. 1974). Enterocytes, necrotic villous tissue, inflammatory cells, and fibrin compose the diphtheritic membrane, and large, gram-positive rods are associated with necrotic areas (Porter 1998). Cholangiohepatitis results when *C. perfringens* gains access to portal circulation and biliary ducts (Lovland and Kaldhusdal 1999).

Virulence Factors. Recent progress in the study of NE, as well as the precedent established by other *C. perfringens*-caused diseases, suggests that pathogenesis is mainly toxin mediated.

Alpha-toxin (CPA). The role of CPA in the pathogenesis of NE is controversial. Results of recent work suggest that a CPA mutant of a virulent chicken isolate retains full virulence *in vivo* (Keyburn et al. 2006), leading to claims that CPA is not involved in pathogenesis. However, there is a substantial body of evidence to the contrary. Alpha-toxin has been detected in feces of poultry, with larger amounts in birds with NE than in healthy birds (Lovland et al. 2003; Gholamiandekhordi et al. 2006), but many early studies of CPA action in the gut of birds with NE were with crude toxin preparations, making it impossible to identify specific CPA effects. Much is known about the CPA action in myonecrosis, but comparison of gut lesions of NE with skeletal muscle lesions of gas gangrene (van Immerseel et al. 2009) yields no information to support or refute a role for CPA in NE. Examination of the effect of CPA on the electrophysiology of the chicken jejunum revealed that it causes electrogenic secretion of anions, probably by stimulation of chloride secretion. Alpha toxin also diminishes electrogenic cotransport of Na+/glucose from the mucosa to the serosa (Rehman et al. 2006), and these findings suggest a pathophysiologic effect that may contribute to signs and lesions of NE. Anti-CPA antibodies are reportedly uncommon in chickens from nondiseased flocks but are found in high titer in birds recovered from NE (Lovland et al. 2003 2004). Newly hatched chicks from older hens have higher anti-CPA antibody titers than those from younger hens (Lovland et al. 2003). Birds inoculated experimentally with *C. perfringens* develop anti-CPA antibody titers (Lovland et al. 2003 2004; Cooper et al. 2008). Finally, anti-CPA antibodies protect against experimental challenge (Kulkarni et al. 2007; Cooper et al. 2008; see below).

NE-toxin. The recently reported NE-toxin (NetB), which occurs widely among NE strains, likely plays a prominent role in the pathogenesis of NE. It has amino acid sequence similarity to CPB (38% identity) and *Staphylococcus aureus* alpha-toxin (31% identity) and causes rounding and lysis of chicken hepatoma cells. A *netB* mutant of a NE strain failed to cause disease, and virulence was restored by complementation (Keyburn et al. 2008). However, about 25% of chicken-virulent strains that were surveyed did not contain *netB* or produce NetB (Keyburn et al. 2008), and it was conceded that NetB might not be an essential component of virulence in all NE strains. Indeed, some birds challenged with the *netB* mutant developed NE, and *netB* negative isolates were obtained from the lesions. It was concluded that the lesions were caused by environmental or endemic *netB* negative strains (Keyburn et al. 2008) and that factors other than NetB likely enable *C. perfringens* to produce NE. Findings of other authors agree (Chalmers et al. 2008; Martin and Smyth 2009; K. K. Cooper and J. G. Songer, unpublished).

Strain dominance. Genetic characterization revealed that type A isolates from groups of healthy birds may comprise five or more pulsed-field types (Engstrom et al. 2003; Nauerby et al. 2003; Gholamiandekhordi et al. 2006; Barbara et al. 2008). However, birds with NE are typically colonized by a single genetic type that is different from those in healthy birds (Engstrom et al. 2003; Nauerby et al. 2003). After natural recovery or treatment, birds again yield multiple genetic types, without the NE strain (Nauerby et al. 2003; Barbara et al. 2008). These effects may be the result of bacteriocin production since NE strains inhibit growth of non-NE strains *in vitro* (Barbara et al. 2008; Timbermont et al. 2009), but, whatever the mechanism, it is likely that strain dominance in NE is a function of the virulence of the organism.

Epidemiology and Risk Factors. A primary risk factor for the development of NE is concurrent infection with coccidia (Hermans and Morgan 2007; Collier et al. 2008). Intestinal mucosal damage by sporozoites and merozoites, together with reductions in intestinal pH and increased transit time, allows establishment and proliferation of *C. perfringens* (Baba et al. 1997). The numbers of *C. perfringens* in the small intestine and ceca of birds with acute coccidiosis are several logs higher than those in birds without coccidia. *C. perfringens* adheres at a higher rate to cecal mucosa in the company of *Eimeria* than when alone. The mortality rate in conventional birds infected with *C. perfringens* and *Eimeria* spp. is typically at least 25% higher than that in *Eimeria*-free birds (Drew et al. 2004). Another major risk factor is establishment, usually by dietary factors, of an intestinal environment that favors anarchic growth of *C. perfringens* (Elwinger et al. 1992; Cooper et al. 2008). Incidence of NE in birds on wheat- or barley-based diets is 6–10 times greater and mortality is 2–3 times greater than those in birds on maize-based diets (Kaldhusdal and Skjerve 1996). This may be due in large part to the availability of preferred nutrients to *C. perfringens* (Annett et al. 2002).

Immunity. Chickens recovered from NE are immune (Thompson et al. 2006), and antibodies from such birds bind to CPA, glyceraldehyde-3-phosphate dehydrogenase, pyruvate-ferredoxin oxidoreductase (PFOR), fructose 1,6-biphosphate aldolase (FBA), and a hypothetical protein (HP), all produced by virulent strains (Kulkarni et al. 2006). All five proteins, prepared as recombinants, immunize broiler chicks against a relatively mild challenge, and responses to CPA, HP, and PFOR protect against a more severe challenge. Immunization with CPA toxoid and boosting with toxin yield the greatest protection against the most severe challenge (Kulkarni et al. 2007). The findings of others are similar (Cooper et al. 2008), yielding significant differences in both incidence and severity of experimentally induced NE. Oral immunization with a *Salmonella* Typhimurium vaccine vector carrying genes encoding FBA or HP also offer substantial protection against challenge (Kulkarni et al. 2008). A similar construct expressing the C terminus of CPA stimulates anti-CPA antibodies when administered to 3-day-old chicks. Lesions resulting from challenge are reduced (compared with controls) in both extent and severity (Zekarias et al. 2008). Thus, there is considerable evidence that immunization with CPA toxoids and other antigens protects to a greater or lesser degree against NE. It remains to solidify these results and to clarify the apparent conundrum that underlies the conflicting viewpoints about the role of CPA and NetB in virulence and immunity.

Porcine Neonatal Enteritis

Clostridium perfringens type A infection remains an important and uncontrolled cause of enteritis in neonatal pigs. Piglets develop creamy-to-watery diarrhea that continues in untreated pigs for about 5 days (Songer 1996). The small intestine is flaccid, thin walled, and often gas filled, with watery contents and no blood. Microscopic lesions can include mild necrotizing enterocolitis and equally mild villous atrophy (Johannsen et al. 1993), but cursory microscopic examination of tissues often reveals no lesions (J. G. Songer, unpublished). However, careful comparison of lesion scores in groups of affected and normal piglets reveals statistically significant differences at the light microscopic level (Songer, unpublished data). Nonetheless, the mildness of the microscopic lesions suggests that type A enteritis is mainly a secretory diarrhea. Jejunum and ileum are heavily colonized with *C. perfringens*, and it is most common to find masses of organisms in the lumen (Johannsen et al. 1993). Capillaries may be dilated, but there is no hemorrhage. Deaths are rare, but the performance of

recovered piglets lags ~10–15% behind that of unaffected piglets. This disease has been reproduced by oral inoculation of gnotobiotic, colostrum-deprived, and conventional piglets (M. A. Anderson and J. G. Songer, unpublished). Unfortunately, despite these studies, little information about virulence factors has come to light. CPA may be involved, but studies to date have been based on the use of crude preparations that contain other toxic molecules. A putative virulence attribute is the beta2-toxin (CPB2; Gibert et al. 1997; Bueschel et al. 2003), which, despite its name, has no significant homology with CPB (Gibert et al. 1997). The initial report suggests that CPB2 is cytotoxic and lethal (for mice), but others have been unable to reproduce these findings (J. G. Songer, unpublished). Strikingly, less than 10% of isolates from normal pigs contain *cpb2*, as compared to >90% among isolates from cases of porcine neonatal enteritis. Thus, while the activity of CPB2 and its specific role in pathogenesis are in question, its strong association with enteric disease in pigs suggests that it is, if nothing else, at least a marker of virulence. CPB2 has an apparent role in typhlocolitis in horses (see below).

Bovine Neonatal Hemorrhagic Abomasitis and Enteritis

Type A infection is a common finding in abomasal ulceration and tympany in beef calves (Mills et al. 1990). Calves with acute onset of abomasal tympany, colic, or death are often suspected of having abomasal displacement or intestinal obstruction (Roeder et al. 1987). Type A is isolated in large numbers from affected tissues, and disease has been reproduced experimentally by inoculation with pure cultures of strains from field cases (Roeder et al. 1988; J. G. Songer, unpublished).

There is little information to link any specific products of *C. perfringens* to virulence for calves. It is common to find CPA in gut contents (J. G. Songer, unpublished), but no objective information ties this toxin to pathogenesis. In our work, *cpb2* was found in only about 50% of isolates from calves with enteritis or abomastitis, and only about half of these produced CPB2 (J. G. Songer, unpublished). However, others have demonstrated a correlation between the occurrence of signs and lesions in Belgian blue calves and production of CPB2 by isolates from affected tissues (Manteca et al. 2002).

Equine Enteritis

Equine neonatal enteritis associated with *C. perfringens* type A infection may be as mild as watery-to-mucoid diarrhea or as severe as peracute death with hemorrhagic mucosal necrosis. Hemorrhagic enteritis is accompanied by subserosal hemorrhage, diffuse mucosal necrosis, and hemorrhage in lamina propria and submucosa. Gram-positive rods that are morphologically compatible with *C. perfringens* are associated with necrotic tissue (Bueschel et al. 1998). There are insufficient data to draw a firm conclusion, but the greatest proportion of these infections may be caused by strains that also bear the enterotoxin gene, *cpe*, and produce CPE. In adult horses, *cpb2* has been associated with about half of a series affected by typical and atypical typhlocolitis. Many of the “consensus” *cpb2* genes from equine isolates had a mutation that terminated transcription and translation at nine amino acids. However, and very interestingly, a ribosomal frameshift was apparently induced in the presence of aminoglycoside antibiotics, resulting in *cpb2* expression and apparently explaining the link between gentamicin treatment and induction of fatal typhlocolitis (Vilei et al. 2005).

Diseases Caused by *Clostridium perfringens* Type B

Clostridium perfringens type B is best known as the etiologic agent of lamb dysentery (table 13.1), which is rare in North America but otherwise occurs worldwide. The source of infection is the dam or her environment, and onset is early in the neonatal period. Nutrient spillover into the small intestine in lambs suckling heavily lactating ewes stimulates anarchic growth of the organism, and inappetance, abdominal pain, and bloody diarrhea develop quickly. Recumbency, coma, and death follow, with a case fatality rate approaching 100%. Extensive small intestinal hemorrhage and ulceration are typical. Similar syndromes may be seen in goats, calves, and foals. “Pine” manifests as chronic abdominal pain, without diarrhea, in older lambs. In the only recent study of type B pathogenesis, production of CPB by isolates of types B and C was found to be similar. The association among ETX, CPB, and CPA production *in vitro* was significant, but data from *in vivo* neutralization studies suggest that CPB and ETX are the most important factors in virulence (Fernandez-Miyakawa et al. 2007).

Diseases Caused by *Clostridium perfringens* Type C

Diseases

Clostridium perfringens type C infects both humans and domestic species worldwide (Songer 1996; table 13.1). Disease is most common in newborns, but may follow alteration of flora (e.g., by sudden dietary changes) in older animals. Disease in young piglets manifests as diarrhea and dysentery (Niilo 1988), with emphysema and comprehensive hemorrhagic necrosis of mucosa, submucosa, and muscularis mucosa (Ohnuna et al. 1992). Case fatality rates approach 100%, often with a peracute clinical course. The clinical course in older piglets is less acute, with nonbloody diarrhea and jejunal mucosal necrosis (Niilo 1988). Sows are the likely source of infection, but numbers in feces may be below the detection threshold of routine methods (Ohnuna et al. 1992). A similar disease occurs in neonatal calves, lambs, foals, and goats. The peracute course of "struck," a form of enterotoxemia occurring in young ewes, leaves the impression of death by lightning strike. Type C invades and multiplies in damaged mucosa, multiplying in the abomasum and small intestine, causing necrosis, without dysentery or diarrhea but with evidence of toxemia.

Pathogenesis

Low concentrations of intestinal proteases in the early neonatal period, as well as the presence of protease inhibitors in colostrum (Niilo 1988), allow the exquisitely protease-sensitive CPB to act in the jejunum. CPB is the primary virulence attribute of type C strains, and its role has been demonstrated by challenge after vaccination with CPB toxoid (Lawrence et al. 1990). Furthermore, log-phase vegetative cultures of type C caused hemorrhagic necrotizing enteritis in rabbit ileal loops (Vidal et al. 2008), and, while a *cpa*/*pfoA* mutant remained virulent in this system, virulence was lost in *cpb* mutants. CPB-neutralizing monoclonal antibodies administered with type C cultures or purified CPB produce the same effect (Sayeed et al. 2008). Findings were similar in an IV-based mouse model (Fisher et al. 2006).

Microscopic and ultrastructural damage occurs as the organism approaches the mucosa, presumably a result of CPB action (Johannsen et al. 1993). Beginning at the jejunal villous apices, the process is progressive, with widespread mucosal necrosis and desquamation of enterocytes. Acute deaths, without diarrhea and likely due to toxemia (Niilo 1988), are common. CPB forms a multimeric complex on human umbilical vein endothelial cells, suggesting oligomerization, and induces release of arachidonic acid and leakage of inositol (Steinthorsdottir et al. 2000). Recombinant CPB forms potential-dependent, cation-selective, 12-Å diameter channels in planar lipid bilayers (Shatursky et al. 2000), channels that are selective for monovalent cations; thus, CPB might function as a neurotoxin, depolarizing membranes of excitable cells. Beta toxin causes plasma extravasation in mice as a result of tachykinin NK1 receptor agonist release. The role of cytokines in this process is related to the release of TNF-alpha via a mechanism involving tachykinin NK(1) receptors but not via TLR4 (Nagahama et al. 2008). Delta-toxin is also produced by type C (and likely by other) strains (Manich et al. 2008). It is a protein of 32.6 kDa with homology to CPB and NetB. Unlike CPB, however, which does not interact with gangliosides, delta-toxin binds to ganglioside GM_2 and forms slightly anion-selective channels.

Diseases Caused by *Clostridium perfringens* Type D

Strains of type D cause enterotoxemia (sudden death, "overeating"), which affects calves, goats, horses, and adult cattle but is most prevalent in young lambs (Uzal and Songer 2008) (table 13.1). Disease is associated with upsets in the gut flora, often from sudden changes to a rich diet or from continuous feeding of a highly concentrated ration (Uzal and Kelly 1997) and is notable for its short clinical course and fatal outcome (Uzal and Kelly 1997). Spillover of nutrients from the abomasum into the small intestine encourages rapid multiplication of organisms and production of ETX. Effects of ETX on the central nervous system (CNS) and other tissues cause sudden death. Hemorrhage is uncommon in ovine and bovine enterotoxemia, but caprine disease frequently presents as chronic hemorrhagic enterocolitis (Blackwell et al. 1991). The variable sensitivity of cell lines to ETX is perhaps a parallel. Madin–Darby canine kidney (MDCK) and human renal leiomyoblastoma (G-402) cells are ETX sensitive, but the concentration of ETX which reduces monolayer viability by 50% is 2 μg/ml for MDCK cells and 280 μg/ml for G-402 cells. Furthermore, the action of ETX is more rapid in MDCK cells exposed to a maximum lethal dose (Shortt et al. 2000).

Epsilon-toxin is one of the most potent bacterial toxins known, with a mouse lethal dose of 100 ng/kg (Gill 1982). The 32.5 kDa inactive prototoxin (E-PTX) is converted to the >1000-fold more toxic form following proteolytic cleavage by host or microbial proteases (Worthington and Mülders 1977). Removal of 13 amino acid N-terminal and 22 amino acid C-terminal fragments is required for activation (Miyata et al. 2002). Small amounts of ETX in the intestine of normal animals are harmless, but persistence of high concentrations leads to increased permeability and absorption into circulation (Bullen 1970). In mice intoxicated experimentally, ETX was absorbed from the small and large intestines but not from the stomach, and lethality was greatest per unit activity when ETX was injected into the colon (Losada-Eaton et al. 2008). The potency of ETX (less than botulinum toxin [1–2 ng/kg IV; Gill 1982] but greater than ricin [2.7 mg/kg IV; Gill 1982]) suggests its potential as a biological weapon and prompted its inclusion as a category B select agent.

Many naturally infected animals die peracutely, without premonitory signs, although some manifest opisthotonus and convulsions. Neurological dysfunction and interstitial and pulmonary edema are produced after administration of ETX to goats and lambs (Uzal and Kelly 1997). Peritoneal and pericardial effusions are common, and hyperglycemia and glycosuria are pathognomonic (Songer 1996). Focal encephalomalacia, with bilaterally symmetrical brain lesions, is a chronic neurological manifestation of enterotoxemia (Buxton et al. 1981). The common name "pulpy kidney" derives from a hallmark lesion in sheep.

Epsilon-toxin binds to detergent-resistant membrane microdomains (or lipid rafts) and oligomerizes to form heptameric complexes in the plasma membrane, causing K^+ efflux and rapid necrosis (Nagahama et al. 1998; Miyata et al. 2002). These findings, and a comparison of amino acid sequences of ETX and other toxins (e.g., aerolysin, perfringolysin O, and *Pseudomonas aeruginosa* cytotoxin), suggest that ETX is a member of a family of pore-forming toxins (Parker et al. 1996). Epsilon-toxin binds with high affinity to rat brain synaptosomes, and the receptor may be a sialoglycoprotein (Payne et al. 1997; Soler-Jover et al. 2007). An ETX-GFP fusion protein was used recently to examine binding of the toxin to tissues of the murine nervous system. Specific binding of ETX to a protein component of myelin in the brain was demonstrated, as was binding to myelinated peripheral nerve fibers. Myelin binding was also demonstrated in tissues from humans and ruminants. Epsilon-toxin strongly stained vascular endothelium in brains of both sheep and cattle (Dorca-Arévalo et al. 2008). Focal-to-diffuse CNS degeneration and necrosis are common in affected animals (Buxton and Morgan 1976). Vascular endothelial tight junctions degenerate (Buxton and Morgan 1976), and perivascular astrocyte processes swell and rupture. Increased capillary permeability, rapid extravasation of fluid (Finnie and Hajduk 1992), and elevated intracerebral pressure follow (Buxton and Morgan 1976). Disruption of the permeability of the blood brain barrier was evidenced by leakage of radiolabeled polyvinylpyrrolidone or serum albumin into the brain.

Permeability effects, evidenced by edema, suggest that ETX is a permease (Worthington and Mülders 1977). MDCK cell toxicity is temperature and pH dependent (Payne et al. 1994) but is unaffected in cells treated with sodium azide or agents which block endosome acidification (chloroquine, monensin, and bafilomycin A1), suggesting that endocytosis or other energy-dependent mechanisms are not required (Petit et al. 1997). On the other hand, MDCK cells exposed to ETX rapidly become permeable to propidium iodide (Petit et al. 2001) and develop morphologic changes consistent with intoxication (Petit et al. 1997 2001). Cells subsequently swell, develop membrane blebs, and lyse (Petit et al. 1997 2001). Binding of ETX to MDCK and rat synaptosomal membranes is associated with the formation of a stable high-molecular-weight (MW) complex (Petit et al. 1997; Nagahama et al. 1998), apparently composed of ETX heptamers (Miyata et al. 2002).

The *etx* gene is located on a plasmid (Cole and Canard 1997) and appears to be associated with IS*1151* (Johnson 1997), suggesting that it may have been mobile. Recent sequencing of an *etx*-bearing plasmid (64.7 kb) revealed *tcp* conjugative transfer genes (Hughes et al. 2007), as well as genes for CPB2, sortase, and collagen adhesin. Most open reading frames identified were also found in an enterotoxin-encoding plasmid from a type A isolate. Different *etx* plasmids were found in other type D strains. The *etx* plasmid may be the result of insertion of *etx*-bearing mobile genetic elements (Miyamoto et al. 2008). The *etxB* gene from a type B strain (Hunter et al. 1992) and *etxD* from a type

D strain differed in only a single amino acid (Havard et al. 1992). ETX is somewhat similar (26% amino acid identity) to the *Bacillus sphaericus* mosquitocidal toxins, sharing similar modes of action and activation by proteolytic cleavage (Liu et al. 1996). Site-directed mutagenesis of HIS106 and HIS149 results in decreased ETX toxicity (Oyston et al. 1998).

Epsilon-toxin stimulates specific antitoxic immunity, and disease control is by vaccination with toxoids (J. G. Songer 1996). Neutralizing and anti-idiotypic monoclonal antibodies have been produced, suggesting that neutralization can be achieved at a single epitope. However, vaccination is not completely protective; anti-ETX titers in vaccinated goats and sheep prevent toxemic deaths in both species, but there is insufficient protection against caprine enterocolitis, in contrast to the much-greater efficacy of immunization in sheep (Blackwell et al. 1991).

Diseases Caused by *Clostridium perfringens* Type E

Iota enterotoxemia in calves and lambs was reported more than 50 years ago, but until recently, most reports dealt with detection of toxin only (Hart and Hooper 1967). At present, type E strains seem to be associated solely with often-fatal hemorrhagic enteritis in neonatal calves (Songer, unpublished). Necropsy findings include abomasal and small intestinal hyperemia and edema, with multifocal mucosal hemorrhage, acute inflammation, and edema of the submucosa. The morbidity rate in affected herds is typically about 10%, with a case fatality rate >50%. About 4% of *C. perfringens* isolates from enteritis in domestic animals are type E, but these represent nearly 50% of all isolates from similar clinical cases in calves (J. G. Songer, unpublished). Strains of type E are distinguished from other toxinotypes (table 13.1) by their production of ITX, which is composed of two noncovalently associated components, Ia and Ib. Ib monomers bind to an unknown cell membrane receptor and move to lipid rafts, where enzymatic component Ia binds to the Ib oligomer (Nagahama et al. 2004). It is internalized but not routed to the Golgi apparatus, and translocation of Ia to the cytoplasm depends on more acidic endosomal conditions (Gibert et al. 2007). Ia then ADP ribosylates actin at Arg-177 (Perelle et al. 1993), which it recognizes via five loops around nicotine adenine dinucleotide (NAD), two of which are essential for ADP ribosyltransferase activity (Tsuge et al. 2008). Iota toxin genes are carried on plasmids, with those described to date being ~97 or ~135 kb in size. Results of plasmid mapping suggest that a mobile genetic element carrying *iA* and *iB* may have inserted into a *tcp*-carrying enterotoxin plasmid (Li et al. 2007).

Silent *cpe* sequences are highly conserved among type E isolates but contain nonsense and frameshift mutations and lack an initiation codon, promoters, and a ribosome-binding site. These strains are not clonal, and the silent type E *cpe* sequences are always located near *iA* and *iB* on episomal DNA (J. G. Songer, unpublished).

CLOSTRIDIUM DIFFICILE

Clostridium difficile was recognized as a human pathogen in 1977 (Bartlett et al. 1978). *C. difficile* infection (CDI) comprises >25% of all cases of human antibiotic-associated diarrhea, at a cost of >$1 billion (Oldfield 2004). Annual costs in Europe are now ~$4 billion per year. CDI has been considered mainly a nosocomial infection (healthcare facility associated [HA] CDI), but epidemiologic dogma is being challenged by a remarkable exponential rise in the rate of community-associated (CA) CDI (Beaugerie et al. 2003). Patients with minimal or no exposure to healthcare settings have been at low risk, but as many as 35% of CA-CDI cases now report no exposure to antimicrobial agents for at least 3 months before onset (Levy et al. 2000). Spores are the key element in transmission of CDI (Gerding et al. 1995; Barbut and Petit 2001) since they may become a permanent part of the environmental “fecal veneer” in hospitals. Colonization of humans also occurs outside the healthcare environment, raising important questions about the source of strains in CA-CDI (Sunenshine and McDonald 2006).

Human CDI often follows disruption of colonic flora by antimicrobials (e.g., clindamycin, ampicillin, amoxicillin, cephalosporins, and fluoroquinolones; Wilcox 1996) and germination of spores in this altered environment (Kelly and LaMont 1998). Vegetative cells produce toxin in the cecum and colon, and the resulting disease may present as diarrhea, colitis, or pseudomembranous colitis (Kelly et al. 1994); fulminant colitis occurs in some patients, with ileus, toxic megacolon, perforation, and death (Kelly and LaMont 1998).

Clinically relevant *C. difficile* strains produce toxins A (TcdA, an enterotoxin) and B (TcdB, a cytotoxin and enterotoxin) or TcdB alone (Poxton 2001) from genes located in the ~20 kb pathogenicity locus (*PaLoc*; Braun et al. 1996). Tissue damage in typical cases is thought to result from the action of these toxins (Kelly and LaMont 1998). TcdA and TcdB belong to the structurally homologous family of large clostridial cytotoxins (Aktories 1997). These toxins monoglucosylate, and thereby inactivate, Rho subfamily proteins, low MW GTP-binding proteins involved in the regulation of the F-actin cytoskeleton (von Eichel-Streiber et al. 1996). Opening of tight junctions and cell death follow disaggregation of polymerized actin. TcdA/B also causes release of proinflammatory mediators and cytokines and activation of the enteric nervous system, leading to polymorphonuclear leukocyte (PMN) chemotaxis and fluid secretion (Johnson et al. 1999). The role in virulence of a distinct binary toxin is not clear (Geric et al. 2006). Toxin production is negatively regulated by TcdC, the product of another gene in the *PaLoc*, which is strongly expressed in the log phase (Hundsberger et al. 1997).

A third toxin (Cdt) is produced by some strains of *C. difficile*, including the hypervirulent strains of ribotype 027 (see below) and the ribotype 078 strains commonly isolated from food animals (Keel et al. 2007). It is a two-component toxin, composed of CdtB (which mediates toxin binding and cell entry) and CdtA (which ADP ribosylates actin) (Barth and Stiles 2008), encoded by the respective genes, which form an operon. No specific role in pathogenesis has been identified for Cdt.

A hypervirulent, epidemic strain has emerged in humans in Canada, Europe, the United Kingdom, the United States, and elsewhere (McDonald et al. 2004). In a single hospital in Quebec, the incidence of CDI in persons over 65 increased by more than eightfold between 1991 and 2003, and the 30-day attributable mortality increased from 4.7% to 13.8%. Experience with this strain (North American pulsed-field type [NAP] 1/ PCR ribotype 027) illustrates how a strain of increased virulence may emerge within, or be introduced into, a population and then spread over a wide geographical area. It may have arisen in the hospital and spread there and into the community. On the other hand, CA-NAP1/027 infection (i.e., in patients not hospitalized within 12 months) accounts for about 1/3 of all NAP1/027 cases, prompting speculation that the epidemic strain may have arisen in the community and been maintained and/or multiplied in human or nonhuman hosts and carried into hospitals by patients or caregivers.

In animals, there is convincing evidence for the importance of *C. difficile* in several species, including horses, swine, and cattle (discussed below). *C. difficile* is also a pathogen of dogs and cats (Marks and Kather 2003; Keel and Songer 2006; Clooten et al. 2007) and is isolated from raw meat intended for pet consumption. Understanding of its role in severe diarrheal illness in other species, not only animals with expanded large intestines, such as hamsters and rabbits, is increasing with the availability of rapid methods to detect toxin in the intestine or feces of animals.

Equine CDI

CDI is an important cause of diarrhea and fatal necrotizing enterocolitis in horses, with profuse watery diarrhea and dehydration in neonatal foals (Weese et al. 2006). Severe hemorrhagic necrotizing enterocolitis, colic, weakness, and dehydration are common, and gram-positive rods line the surface of necrotic villi. The clinical course is usually less than 24 h, not uncommonly with fatal outcome. Disease in foals has been reproduced experimentally (Arroyo et al. 2004). *C. difficile* has become important as a cause of nosocomial, often-antimicrobial-associated, diarrhea in adult horses. Among adult horse isolates, most carried both *tcdA* and *tcdB*, although some had only *tcdB*. More than 25% were nontoxigenic (Arroyo et al. 2007). The organism may also be involved in the etiology of duodenitis-proximal jejunitis (Arroyo et al. 2006).

Porcine CDI

We documented the emergence of porcine neonatal CDI (Songer et al. 2000; Songer 2004), in which the case definition typically includes animals 1–7 days of age, presenting with a history of "scours from birth." Moderate to severe mesocolonic edema and pasty-to-watery, yellowish colonic contents are common. Some cases present with mild-to-severe systemic signs. Diagnosis is by toxin detection (Keel and Songer 2006). On average, 2/3 of litters and 1/3 of individual pigs in an infected farrowing barn are affected, but rates may be as high as 97% (Songer, unpublished). Weaning weights are ~1–2 lb per pig less than average. Bacteriological culture yields heavy growth of *C. difficile* from colon, but

diagnosis is based upon detection of TcdA and B. CDI is reproduced with pure cultures of *C. difficile* (CD) (M. A. Anderson and J. G. Songer, unpublished), including the human epidemic strain NAP1/027. Infection may occur beyond the neonatal period (Songer, unpublished), and CDI in mature pigs can take a severe form similar to that in some human cases (Kiss and Bilkei 2005). Age-stratified sampling in a 13,000 sow herd revealed CD in 1/3 of normal nursery pigs and >15% of normal finishing pigs (J. G. Songer, unpublished).

Bovine CDI

There is increasing evidence that CD is associated with enteritis in young calves. Rodriguez-Palacios et al. (2007) conducted a case-control study in calves (mean age 14.2 days) from 102 dairy farms in Canada. *Clostridium difficile* was isolated from 7.6% of diarrheic calves (n = 144) and 14.9% of controls (n = 134) (p = 0.009). Toxins were detected in 39.6% of calves with diarrhea and 20.9% of controls (p = 0.0002). Feces (n = 253) from diarrheic calves 1 day to 6 weeks of age in southwestern calf ranches were culture positive (>20%) and/or toxin positive (23.3%), with an overall positivity rate of 33.6%. Tissues from affected calves had inflammatory cell infiltrates in lamina propria of the upper colon, and occasional crypts were distended with sloughed epithelial cells and degenerate neutrophils. Mild epithelial degeneration and detachment were noted in the mid- and lower colon, with foci of shrunken and pyknotic cells overlaid with fibrin. Neutrophil infiltration is in keeping with CDI in other species (Keel and Songer 2006; Hammitt et al. 2008). Large gram-positive rods morphologically compatible with CD were abundant in the proximal and distal colonic lumen. Lesions produced by inoculation with TcdA, TcdB, or both were similar to those in other species inoculated with toxins or organisms (Keel and Songer 2006). Despite the association of toxigenic CDI with enteric lesions typical of CDI, the final proof that this organism causes enteric disease in cattle would be experimental reproduction of the disease. To date this has not been achieved (Rodriguez-Palacios et al. 2007).

Characterization of calf isolates (Rodriguez-Palacios et al. 2006) revealed strains of seven ribotypes that occur in humans. Among these, ribotypes 017 and 027 have been associated with severe disease (Warny et al. 2005), and lack of geographic clustering of ribotype 027 strains may indicate wide dissemination in the community in different animal species. These strains are also fluoroquinolone resistant, as are human strains, strengthening speculation about interspecies transmission.

Four ribotypes were identified among neonatal pig isolates (n = 144) from Iowa, Ohio, Montana, North Carolina, Texas, and Utah (Keel et al. 2007), but ribotype 078 accounted for 83% of swine isolates. Strains of ribotype 078 were also predominant in the calf studies described above (Hammitt et al. 2008). Ribotype 078 strains were also uniformly PCR positive for binary toxin gene *cdtA* and carried a 39 bp deletion in *tcdC*. Many piglet strains are more than 80% similar to strains from human *C. difficile*-associated diarrhea (CDAD) (Jhung et al. 2008), including some fatal cases. Increasing numbers of ribotype 078 and other toxinotype V strains are being found in the United States and the United Kingdom.

Recently, *C. difficile* has been isolated from uncooked and ready-to-eat retail meats (Rodriguez-Palacios et al. 2007; Songer et al. 2009), and many of the strains are of ribotypes that are also associated with CDI in humans and food animals. Transmission from food animals to foods to humans has not yet been documented.

Immunity

Little attention has been given to immunoprophylaxis of CDAD in domestic animals, although effective immunity will probably be found to be antitoxic. In mouse and hamster models, antibodies against TcdA prevent toxin binding, eliminate secretion and inflammation, and prevent clinical disease (Allo et al. 1979). Anti-TcdB antibodies also participate in protection against CDAD (Kink and Williams 1998). More than 60% of adults and older children have antibodies against TcdA and TcdB (Kelly et al. 1992), perhaps due to repeated intestinal exposure. However, protective immune responses arise only following severe or relapsing infections. Antitoxic antibody concentration in serum is inversely proportional to the severity of disease and risk for relapse (Kelly et al. 1992; Warny et al. 1994). Convalescent sera contain TcdA-neutralizing IgG and IgA, and rising titers of antiTcdA antibodies correlate with the resolution of clinical disease (Warny et al. 1994). Active immunization against TcdA prevents clinical disease (Kim et al. 1987) and anti–TcdA antibodies protect passively (Allo et al. 1979; Leung et al. 1991). Thus, systemic antiTcd

IgG and IgA, as well as mucosal IgA, are involved in protection against CDAD.

CLOSTRIDIUM SEPTICUM

Diseases

The most notable enteric infections caused by *C. septicum* affect ruminants and can be grouped loosely under the name “braxy” (Songer 1996; table 13.2). The organism establishes in the abomasum, causing extensive local damage and often producing a fatal bacteremia (Saunders 1986). The pathogenetic mechanism may involve mucosal damage following ingestion of frozen feed (Ellis et al. 1983; Saunders 1986; Schamber et al. 1986). Abomasal walls and the proximal small intestine become edematous, hemorrhagic, and necrotic (Ellis et al. 1983). Braxy is most common in calves (Saunders 1986), and humans with hemolytic uremic syndrome (HUS, *Escherichia coli* O157:H7 infection) may become superinfected by *C. septicum*, developing highly fatal necrotizing enterocolitis (Barnham and Weightman 1998).

C. septicum is found in soil and in feces of domestic animals and humans. Postmortem invasion from the digestive tract is common, particularly in ruminants. The organism can also enter animals in one of the life stages of liver flukes (Songer 1996), after the manner of *Clostridium novyi* types B and D. Iatrogenic infections are not uncommon in horses.

Pathogenesis

Alpha-toxin is the only lethal virulence attribute of *C. septicum* (Ballard et al. 1992), with a mouse LD_{50} of ~10 µg/kg. It is secreted as a 46.4 kDa protein (Ballard et al. 1992; Ballard et al. 1995). The primary structure of alpha-toxin has ~72% similarity with that of aerolysin (from *Aeromonas hydrophila*), and its mode of action is also similar (Howard and Buckley 1985; Ballard et al. 1993). The amino terminal domain of mature aerolysin (~70 amino acids) is absent from alpha-toxin (Parker et al. 1994; Ballard et al. 1995). A C-terminal propeptide may be a chaperone, stabilizing alpha-toxin monomers and escorting them to the membrane (Ballard et al. 1993; Sellman and Tweten 1997). The cleavage site is rich in basic amino acids and has a consensus site for the cell surface protease furin (Gordon et al. 1997). The cleaved propeptide remains associated with the toxin and is displaced when activated monomers interact after binding to cell surface receptors, including glycosylphosphatidylinositol-anchored proteins (Sellman and Tweten 1997; Sellman et al. 1997; Gordon et al. 1999). Bound, activated monomers interact via lateral diffusion to initiate formation of the oligomeric prepore complex. Transmembrane regions of the complex insert, forming the beta-barrel structure and the membrane pore (Sellman and Tweten 1997). Deletions in the transmembrane domain of alpha-toxin eliminate pore formation and any contribution of the toxin to pathogenesis. Vascular endothelial cytotoxicity may result in loss of fluid from circulation and induction of shock.

CLOSTRIDIUM SPIROFORME

Clostridium spiroforme is the cause of iota enterotoxemia of rabbits and other laboratory rodents (Carman and Borriello 1984). This organism has a distinct, loosely coiled, spiral form when grown on blood agar. The coils consist of a uniform aggregation of numerous individual semicircular cells joined end to end.

Diarrhea caused by *C. spiroforme* occurs spontaneously in weaned rabbits (Katz et al. 1978), but destabilization of cecal microflora at weaning or by antimicrobial therapy is commonly involved in the initiation of disease. Diarrhea with perineal staining develops quickly, and death is common. The cecum

Table 13.2. Enteric Infections by Clostridia Other Than *C. Perfringens*

Species	Virulence factor(s)	Diseases
septicum	Alpha (pore former)	Abomasitis (braxy) in sheep, calves
difficile	A (enterotoxin), B (enterotoxin, cytotoxin)	Antibiotic-associated typhlocolitis (humans, rodents), equine neonatal hemorrhagic enterocolitis, porcine neonatal typhlocolitis
piliforme	Undefined toxin	Various species: enterohepatic infection (Tyzzer’s disease)
spiroforme	*spiroforme* toxin	*iota*enterotoxemia (rabbits, foals?)

becomes immensely dilated, with watery contents, and epithelial necrosis is accompanied by pronounced inflammation of the lamina propria. Affected animals have iota-like toxin and high numbers of spores in cecal contents. Isolates of *C. spiroforme* produce a toxin that is neutralized by serum against *C. perfringens* iota toxin. Toxin produced *in vitro* is lethal and dermonecrotic, and enterotoxemia can be reproduced with filtrates of cecal contents from rabbits that have died of the disease. ADP-ribosyltransferases (toxin component Ia) of 43–47 kDa MW have been purified from *C. spiroforme*. These have no effect when examined alone *in vivo* or *in vitro*, but lethal and cytotoxic effects occur when proteins with transferase activity are mixed with trypsin-activated toxin component Ib (Popoff and Boquet 1988). ADP-ribosylation of monomeric actin causes cytoskeletal damage. Rabbits immunized with an iota toxoid are protected against IP toxin challenge (Ellis et al. 1991).

CLOSTRIDIUM PILIFORME

Clostridium piliforme causes enterohepatic disease (Tyzzer's disease) in many animal species (van Andel et al. 1998 2000), including immunocompromised humans (Smith et al. 1996). Disease in laboratory rodents is commonly subclinical, although mouse strains differ in susceptibility to disease (Waggie et al. 1981). The organism is unlike other clostridia in that it is obligately intracellular, gram negative, and produces lipopolysaccharide (van Andel et al. 1998). Sequencing of 16S and 23S rRNA genes and the intergenic spacer and subsequent phylogenetic analysis revealed that *C. piliforme* forms three clusters within a single clade among 45 related bacterial species. Its nearest relative was *C. colinum*, the cause of ulcerative enteritis (quail disease; Feldman et al. 2006).

Tyzzer's disease can also manifest as a rapidly progressive hepatitis of foals. Lethargy, recumbency, seizures, and fever are observed during a mean clinical course of 30 h. Affected foals also experience metabolic acidosis, hypoglycemia, and elevated hepatobiliary enzyme activity. The case: fatality rate is high (Borchers et al. 2006). Microscopic lesions include multifocal coagulative necrosis and hepatitis, and filamentous bacilli are found in cytoplasm of hepatocytes. Occurrence may be seasonal, and foals born to mares less than 6 years of age are at significantly greater risk (Fosgate et al. 2002).

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Recent findings have greatly increased our understanding of clostridial enteric disease. Insights into molecular mechanisms of action of *C. perfringens* CPB and ETX and *C. septicum* alpha-toxin provide opportunities for more extensive work on the interaction of these organisms with their hosts. The same can be said for the emergence and expansion of the host range of *C. difficile*. On the other hand, our knowledge of the mechanisms by which clostridia interact with their hosts is barely beyond embryonic. Many aspects of clostridial biology in the gut (such as possible biofilm behavior and the role of acid resistance) present opportunities for research, to the benefit of livestock production and basic understanding of the interaction of bacterial pathogens and hosts. Experimental reproduction of clostridial enteric disease is an especially important area of endeavor.

Data from genome sequences will provide a basis for more-detailed characterization of toxins and other virulence factors, as well as discovery of novel mechanisms of pathogenesis. It seems likely that genomic sequencing will allow us to identify the pathogenome for strains affecting various species. The relatively small community involved in research on clostridial disease in domestic animals is highly productive but too small to make rapid progress.

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14
Salmonella

P. A. Barrow, M. A. Jones, and N. Thomson

INTRODUCTION

Salmonella remains a human and animal pathogen of worldwide significance, and it has remained at the center of microbiology for several decades, with frequent reviews of progress (Neidhardt et al. 1996; Wray and Wray 2000).

On the basis of pathogenesis and infection biology, *Salmonella* serovars can be classified by association with and production of at least three distinct types of infection (pathovars). A small number of serovars are capable of producing severe systemic disease in immunologically and physiologically normal, outbred, healthy adult individuals of a narrow range of animal species. Transmission is generally via the fecal–oral route, and bacterial multiplication is widely considered to take place primarily in the cells of the macrophage monocyte lineage. In the target species, the alimentary tract becomes involved pathologically only in the later stages of the disease, and thus, in the absence of disease little intestinal colonization takes place. Such infections are associated with *S*. Typhi and Paratyphi A and some strains of Paratyphi B, which produce typhoid in man, *S*. Gallinarum in poultry and probably other birds, and *S*. Choleraesuis in pigs. A second group of serovars are associated with systemic infections, with a focus of infection frequently involving the reproductive tract when animals are pregnant (mammals) or in lay (birds) and may also be involved in more extensive systemic multiplication and enteritis in very young animals. These include *S*. Dublin in cattle, *S*. Abortusovis in sheep, *S*. Abortusequi in horses, and *S*. Pullorum in birds.

The vast majority of the remaining serovars are unable to produce systemic infections in normal, healthy adult animals. They are, however, able to colonize the alimentary tract of a range of animals, and may cause acute enteritis or subclinical infections. The molecular basis of *Salmonella*-induced enteropathogenesis is only now becoming better understood (see below). As a consequence of intestinal colonization and high levels of fecal shedding, particularly in food animals, bacteria can enter the human food chain, infect the gut, and cause gastroenteritis.

A classification of *Salmonella* virulence may also be made on the basis of host-range, although there is disagreement over the terminology. In one school of thought, association with host species is the basis for division into three groups. *Host-specific* serovars typically cause systemic disease in a limited number of phylogenetically related species, as indicated above. Thus, *S*. Typhi, *S*. Gallinarum, and *S*. Abortusovis are almost exclusively associated with systemic disease in humans, fowl, and sheep, respectively. *Host-restricted* strains, while primarily associated with one or two closely related host species, may also infrequently cause disease in other hosts. For example, *S*. Dublin and *S*. Choleraesuis are generally associated with severe systemic disease in ruminants and pigs, respectively. In the United Kingdom, between the years 1958 and 1967 (the most recent years for which meaningful epidemiological data are available), analysis of the incidence of salmonellosis in food animals demonstrated that 99% of all *S*. Choleraesuis incidents were associated with pigs and 95% of all *S*. Dublin incidents were associated with cattle. In contrast, the *ubiquitous*

serovars, such as *S*. Typhimurium and *S*. Enteritidis usually induce gastroenteritis in a broad range of unrelated host species.

Another school of thought divides the serovars into the two main groups producing different pathological scenarios (pathovars): those that typically produce systemic disease and those that colonize the intestine and produce enteritis. In this model, the ability to produce typical typhoid-like infections is restricted to those serovars that produce disease either in mammals (*S*. Dublin, *S*. Typhimurium, *S*. Enteritidis in mice, *S*. Choleraesuis in a wide range of mammalian species, and *S*. Typhi and Paratyphi, restricted to man) or in avian species (*S*. Gallinarum and its related serovar, *S*. Pullorum). The experimental evidence tends to support this classification with some provisos. Thus, the fact that typical typhoid is only produced in mice by selected group B and D serovars (*S*. Typhimurium and *S*. Enteritidis) suggests an increased susceptibility in certain strains of mice. *S*. Choleraesuis is highly virulent and produces typhoid experimentally in mice, rats, guinea pigs, rabbits, and also calves. In contrast, *S*. Typhi and Paratyphi produce typical infection only in man. All these serovars never produce typical infection in adult birds. The contrary is also true, that *S*. Gallinarum and *S*. Pullorum never produce typical typhoid in mammals.

There appears to be no clear host species association based on ability to cause enteritis. Strains of *S*. Typhimurium produce enteritis in a variety of adult mammals including man. The situation with other serovars is less clear, although a variety of serovars produce enteritis in the young of several species including cattle, pigs, sheep, companion animals, and poultry.

Age has an enormous effect on disease susceptibility. Typically, very young animals are highly susceptible to *Salmonella* infections as a result of both a simple noninhibitory gut flora which takes some time to mature and immunological immaturity. Variation in disease can be observed which shows clearly that the age of the animal at infection is important in development of disease. *Salmonella enterica* infection of young chickens of less than 3 days old can lead to severe disease (Barrow et al. 1987); in contrast, no clinical signs are observed with infection of healthy adult chickens (Barrow and Lovell 1988). Although this was originally attributed to macrophage maturity, it is now thought more likely to be the result of neutrophil/heterophil granulocyte maturity and numbers. Thus, depletion of mature chickens of heterophils using 5-fluorouracil induced a degree of susceptibility to systemic salmonellosis normally seen only in newly hatched birds (Kogut et al. 1993).

Some of the factors associated with age are poorly defined and understood. Thus, genetic differences in susceptibility to intestinal colonization of chickens by *Salmonella* are clear when the birds have a mature gut flora and are at least 6 weeks of age, but these differences are not expressed until the birds reach this age (Barrow et al. 2003; Beal et al. 2004).

SALMONELLA INFECTIONS OF CATTLE

Salmonellosis in cattle occurs worldwide and is associated primarily with serovars Dublin and Typhimurium. During the past 20 years, approximately 100 serovars other than *S*. Dublin and *S*. Typhimurium accounted for less than 10% of incidents in the United Kingdom. *S*. Dublin and *S*. Typhimurium are endemic in northern Europe, although the distributions of these serovars differ. In the United Kingdom, *S*. Typhimurium occurs in all geographical regions, whereas *S*. Dublin is predominately found in north- and southwestern England and Wales. In the United States, *S*. Typhimurium is endemic in cattle throughout the country. In contrast, *S*. Dublin, which before the 1980s occurred only to the west of the Rocky Mountains, has recently spread eastwards to other states and north into Canada from where it had not previously been isolated.

Salmonellosis reached a peak in the British cattle industry in the 1960s, with over 4000 incidents in 1969, most of these being associated with infections in young calves, predominantly involving *S*. Dublin. More recently, there has been a steep decline in the number of *Salmonella* outbreaks, and over the last 5 years there have been only 400–500 incidents per annum, with similar numbers of incidents caused by *S*. Dublin and its replacement, *S*. Typhimurium. Epidemiological analysis of *S*. Dublin by electrophoresis indicated three phenotypes, one of which is global in distribution and in which, in the United States, nonmotile strains are common. Vi-producing strains were restricted to one of the taxa, isolated from the United Kingdom and France at that time. Phage typing of *S*. Dublin also indicates that a small number of types predominate. This, together with

the fact that most infections are limited to within herds may, to some extent account for the absence of multiple antibiotic resistance in *S*. Dublin which is frequent in *S*. Typhimurium.

In *S*. Typhimurium, a small number of strains also tend to predominate. These tend to be multiresistant; PT 29 in the 1960s, 204c in the 1990s, and DT104 until recently.

Infected cattle may excrete up to 10^8 colony-forming units (CFU) *Salmonella* per gram of feces, and environmental contamination is thus a potent source of infection. Subclinical excretion of *Salmonella* occurs in which cattle excrete *Salmonella* in concentrations greater than 10^5 CFU/g of feces. Active carriage is usually the sequel to clinical enteritis or systemic infection, and infected animals may excrete *Salmonella* for years or even for life. In some animals, known as "latent carriers," *Salmonella* persists subclinically in the tissues but are only intermittently excreted in feces. Excretion may be activated by stress, for example, at parturition or following *Fasciola hepatica* or *Babesia* infection.

Salmonellosis in Calves

In calves, clinical disease is most common at 2–6 weeks of age. Clinical signs vary, but typically the enteric form of disease predominates which is characterized by pyrexia, dullness, and anorexia, followed by diarrhea that may contain fibrin and mucus. Pneumonia may also occur. The feces may become blood stained and "stringy" due to the presence of necrotic intestinal mucosa. Calves rapidly become weak and dehydrated, and unless treated, infected calves usually die 5–7 days after the onset of disease. At this stage, the organism has become systemic, probably largely as a result of reduced innate immunity, and may be isolated from a variety of tissues, including the blood. Calves that recover from infection do not typically remain carriers.

Salmonellosis is very variable and in some animals, particularly the very young, rapid multiplication occurs both in the intestine and systemically, associated with poor absorption of specific IgG from colostrum or with calves receiving insufficient or no colostrum. This may be accompanied by true septicemia, although this has never been accurately determined, and animals may die in a comatose state after 3–5 days in the absence of diarrhea.

The breed of animal can also affect the outcome with some breeds, such as Jersey, being far more susceptible than Friesian and beef breeds. Similarly, preinfection with parasites such as *F. hepatica* in experimental rat infections with this parasite or the nematode *Nippostrongylus brasiliensis* increases the severity of disease.

Salmonellosis in Adult Cattle

In adult cattle, both acute and subacute forms of disease are recognized, caused by *S*. Dublin or, less frequently, *S*. Typhimurium or other serovars including *S*. Enteritidis. The onset of the severe, acute form of disease is sudden and typically accompanied by pyrexia, dullness, anorexia, and reduced milk-yield. Severe diarrhea follows, which may contain blood, mucus, and necrotic intestinal mucosa. Early in infection, *Salmonella* may be isolated from blood and milk in addition to the feces. Pregnant animals generally abort following *S*. Dublin infection. High temperatures usually persist for several days and then fall just prior to death (1–5 days after infection). Abortion may occur in the absence of other clinical signs, and this is particularly the case with *S*. Dublin infections.

SALMONELLA INFECTIONS OF SHEEP

Compared with bovine salmonellosis, the general incidence of disease in sheep in most countries is low, reflecting their generally less intensive management. In many countries, including Mediterranean countries, disease caused by the host-adapted strain *S*. Abortusovis remains an important economic problem. In many other countries, it is a relatively minor problem and other serovars, such as *S*. Typhimurium, are predominant. *S*. Dublin may also produce disease in sheep. Currently, the most frequently isolated serovars are *S*. Typhimurium (primarily DT104), *S. arizonae* (O61:k:1,2,7), *S*. Derby, and *S*. Montevideo, but the list is by no means restricted to these. *S*. Montevideo has been associated with abortion in ewes for a number of years. This is caused largely by a single biotype, which is different from that causing infections in man, cattle, and poultry. In the United States, Canada, and some European countries, *S. arizonae* (O61:k:1,5,7) is an important serovar.

In comparison with bovine salmonellosis, there is much less information on the routes of transmission. *S*. Abortusovis may be isolated from the genitalia of both female and male sheep, but the extent to which transmission occurs by this route and is responsible

for infection is disputed. In the case of *S. arizonae*, nasal carriage also appears to be an important aspect of the epidemiology.

As with *S.* Dublin infection in cattle, other factors may precipitate disease in carriers. These include *Chlamydia* infection, chemoprophylaxis, nutritional factors including drought, and other factors inducing stress, such as cold and overcrowding.

The course of infection is similar with *S.* Abortusovis and *S.* Montevideo, with mortality varying between 10% and 75%. In adult animals, signs are variable and may be few prior to abortion. Profuse growth of *S.* Abortusovis may be obtained from embryonic tissues. This generally does not appear to affect the ewe greatly which may lamb normally in the next season and after abortion, excretion is short lived. Lambs may also be stillborn and may die soon after birth, or septicaemia may occur during the first few weeks of life, although little is known of the pathogenesis of these infections. In addition, *S.* Montevideo may also be excreted in the feces without abortion.

S. Typhimurium and *S.* Dublin may produce largely enteric infections, with systemic involvement, depending on a number of additional factors, such as age, nutrition, and stress. *S.* Abortusovis is invasive for ovine epithelial cells *in vitro* and *in vivo*; this property is dependent on its type three secretion system (TTSS) 1. The colonization of ovine intestinal and systemic tissues by *S. enterica* serovars with different host specificities has been determined in 1- to 2-month-old lambs. Following oral inoculation, serovars Abortusovis, Dublin, and Gallinarum were recovered in comparable numbers from the intestinal mucosa, but serovar Gallinarum was recovered in lower numbers than the other serovars from systemic sites. The pattern of bacterial recovery from systemic sites following intravenous inoculation was similar. Intestinal invasion was quantified in ovine ligated ileal loops and serovars Dublin, Gallinarum, and Typhimurium were recovered in comparable numbers, whereas serovar Abortusovis was recovered at concentrations approximately 10-fold lower. *S.* Typhimurium and *S.* Dublin induced intestinal inflammatory responses, whereas mucosae infected with serovars Abortusovis and Gallinarum were indistinguishable from uninfected mucosae. Together these data suggest that *Salmonella* serovar specificity in sheep correlates with bacterial persistence at systemic sites (Barrow et al. 1994). Intestinal invasion and avoidance of the host's intestinal inflammatory response may contribute to but do not determine the specificity of serovar Abortusovis for sheep. In common with some other systemic serovars, cytokine profiles differ from those of animals infected with serovars which are not host specific. Thus, in contrast to *S.* Dublin TNF-α and IL-12 transcription is reduced in the spleen and draining lymph node following subcutaneous challenge with *S.* Abortusovis (Montagne et al. 2001).

SALMONELLA INFECTIONS OF PIGS

The serovars of *Salmonella* associated with clinical disease in pigs can be divided into two groups: the host-restricted serovars typified by *S.* Choleraesuis and the ubiquitous serovars typified by *S.* Typhimurium. Since the 1960s, the occurrence of *S.* Choleraesuis has fallen dramatically in the United Kingdom and is now only isolated sporadically, whereas it remains a major threat to the pig industry in the United States. *S.* Derby also has a strong association with pigs in both countries. A survey of healthy pigs at slaughter in abattoirs in the United Kingdom showed that 23% of pigs had *Salmonella* culture-positive cecal contents in which *S.* Typhimurium and *S.* Derby were the predominant serovars.

Oral ingestion is thought to be an important route of infection as *Salmonella* are shed in high numbers in the feces of clinically infected pigs, although high doses of between 10^8 and 10^{11} CFU are required to cause disease experimentally. Pneumonia is a common feature of *S.* Choleraesuis infections in pigs, and several studies have shown that pigs can be experimentally infected by intranasal inoculation. The tonsils and lungs are thought to be important sites of invasion.

Clinical salmonellosis in pigs generally takes two forms: septicemia caused by host-restricted serovars such as Choleraesuis, and enterocolitis caused by broad host-range serovars such as *S.* Typhimurium. Not surprisingly, weaned pigs that are intensively reared are most frequently affected by *Salmonella* infections. *S.* Choleraesuis has the capacity to cause disease in both young and older animals, whereas *S.* Typhimurium typically causes disease in pigs aged between 6 and 12 weeks, but rarely in adult animals. In older animals, subclinical infections with *S.* Typhimurium are frequent, leading to high transmission rates if active carrier animals are not detected.

Pigs infected with *S*. Choleraesuis are lethargic, pyrexic, and often with respiratory symptoms, including coughing. Diarrhea may or may not be present, and cyanosis of the extremities is common. In most cases, mortality is high. *S*. Typhimurium typically causes enterocolitis with a watery diarrhea. Pigs become anorexic, lethargic, and febrile, but mortality is typically low. Pigs are frequently associated with acute and subclinical infections caused by other serovars of *Salmonella*.

The different forms of porcine salmonellosis can be reproduced experimentally. There is some evidence that serovars Typhimurium and Choleraesuis may invade the intestinal mucosa by distinct routes. Following oral infection, *S*. Typhimurium shows low invasiveness for the enteric mucosa and does not reveal any tropism for a specific intestinal location. However, *S*. Choleraesuis is found predominantly in colon and on the luminal surface of ileal M cells of Peyer's patches. More recently, it was reported that serovars Choleraesuis and Typhimurium invaded enterocytes, goblet cells, and M cells in porcine ileal mucosa, but that serovar Choleraesuis was found more frequently within M cells than *S*. Typhimurium. In addition, serovar Choleraesuis appeared to induce less damage to the mucosa than did Typhimurium (Meyerholz and Stabel 2003), which is consistent with the theory that host-specific serovars cause systemic disease by a strategy of stealth (*vide infra*).

INFECTIONS OF DOMESTIC FOWL AND OTHER AVIAN SPECIES

The prevalence of *Salmonella* serovars in domestic fowl varies in different countries and with time, with serovars emerging for a period of time and then disappearing with no obvious cause. In the 1950s, *S*. Agona was prevalent in U.K. poultry industries, yet disappeared without any obvious intervention. Historically, *S*. Typhimurium has been among the most prevalent serovars isolated from poultry. In the United Kingdom, between the years 1968 and 1973, *S*. Typhimurium accounted for over 40% of all *Salmonella* isolations associated with poultry, followed by *S*. Enteritidis (6%), *S*. Pullorum (4%), and *S*. Gallinarum (3%). During this time, *S*. Hadar became established in the U.K. poultry industry, initially in turkeys and then also in chickens. During the 1980s, *S*. Enteritidis phage type 4 (PT4) emerged as the predominant serovar, exceeding the isolation rates of *S*. Typhimurium. After *S*. Typhimurium and *S*. Enteritidis, the most commonly isolated serovars in the United Kingdom are *S*. Livingstone, *S*. Senftenberg, *S*. Kedougou, and *S*. Montevideo.

The high prevalence of *S*. Enteritidis, *S*. Typhimurium, and other serovars in poultry is reflected in many other parts of the world. There are some exceptions; for example, *S*. Enteritidis is virtually absent from Australasia. This is likely to be due to a result of strict controls on importation of poultry. However, these countries have their own epidemiological peculiarities, such as the high incidence of *S*. Sofia in poultry. Interestingly, this is not reflected in a high incidence of *S*. Sofia infections in humans.

The poultry-specific serovars *S*. Gallinarum and *S*. Pullorum have largely been eradicated from the poultry industries of Europe and North America. However, in regions of the world with less developed industries, and particularly in facilities with poor biosecurity, these serovars still represent major threats to bird health and welfare and the local economy. Although chickens are the most economically important hosts for *S*. Gallinarum and *S*. Pullorum, natural outbreaks caused by these serovars have been described in turkeys, guinea fowl, and other avian species. In addition, the increase in the popularity of free-range rearing in northern Europe has resulted in an increase in incidence of *S*. Pullorum infection in poultry.

The sources of infection in poultry are many, including poultry themselves, feed, and the environment and in the cases of *S*. Gallinarum, *S*. Pullorum, and *S*. Enteritidis via vertical transmission.

Fowl typhoid and pullorum disease are systemic diseases that occur primarily in chickens and turkeys but are also important in game birds. Fowl typhoid is caused by *S*. Gallinarum and pullorum disease by the serologically identical *S*. Pullorum. Both these serovars are highly host specific and are rarely associated with systemic disease in nonavian species. Fowl typhoid is widely considered to be a disease of adult birds and pullorum disease a disease of chicks and poults; however, *S*. Gallinarum is able to infect both young and older birds.

Horizontal transmission of *S*. Gallinarum is largely fecal–oral, and the organism colonizes the gut very poorly and soon disappears from the feces, but is taken up, primarily by the cecal tonsil and Peyer's patch, as indicated by higher bacterial counts soon after oral infection. *S*. Gallinarum

bacteria are then soon found in low numbers in organs rich in the monocyte-macrophage cell lineage, mainly spleen and bone marrow, where it is thought that they multiply. After bacterial counts have increased considerably at these sites, a bacteremia occurs and the organisms are then found in localized areas in the intestinal wall, possibly in aggregates of lymphoid cells. From these sites, they are shed into the intestine in large numbers, although how this occurs is not known. Surviving birds show large areas of necrosis in the myocardium, from which, unlike the case with *S*. Enteritidis, it is not possible to isolate viable bacteria. Older literature, involving epidemiological studies, suggests that vertical transmission of *S*. Gallinarum is an important mode of spread. However, it is very difficult to reproduce this experimentally (Berchieri et al. 2001).

S. Pullorum is regarded by many as a biotype of *S*. Gallinarum, although multilocus sequencing indicates that both have a common ancestor (Li et al. 1993), and more recent work on the glycogen biosynthesis genes indicates that in *S*. Pullorum the sequence of *glgC*, encoding ADP glucose pyrophosphorylase, more closely resembles that of *S*. Typhimurium than *S*. Gallinarum (McMeechan et al. 2005). However, the organism is less virulent in older birds than *S*. Gallinarum. High mortality occurs only when birds are infected within a few days of hatching. After this time no disease occurs, and like *S*. Gallinarum in older birds, it colonizes the intestine very poorly. However, in the absence of a mature gut flora, infection soon after hatching results in massive bacterial replication within the intestines with an associated enteritis. How the organism becomes systemically disseminated is uncertain, but bacterial multiplication is thought to take place in the spleen and liver with an ensuing septicemia. Mortality is strain and host background dependent. In surviving birds, the organism persists in the spleen and despite high levels of circulating antibody, it is not eliminated for months. When the hens become sexually mature at 16–20 weeks, the organism multiplies in the tissues again and spreads to other sites, including the reproductive tract, and particularly the ovaries, from where eggs may become infected (Wigley et al. 2001). The tropism of *S*. Pullorum for the reproductive tract is much greater than is the case for the ubiquitous serovars. *S*. Pullorum readily infects the reproductive tract and developing eggs following oral infection, with particularly high numbers in the oviduct at the point of lay (Wigley et al. 2001). The molecular basis for this tissue tropism remains unknown.

The capacity of serovars other than Gallinarum and Pullorum to cause disease is relatively poorly understood. *S*. Typhimurium, *S*. Enteritidis, and some strains of other serovars are capable of producing clinical salmonellosis in very young birds, probably by a mechanism common to that of *S*. Pullorum. The reasons why serovars such as Heidelberg, Seftenberg, Infantis, Montevideo, and Menston are apparently much less virulent in chicks yet retain the capacity to colonize the intestine and cause human food poisoning are not understood, although these latter strains do not possess the virulence plasmid.

The potential for *Salmonella* to invade the intestines of poultry has been assessed in several studies. The passage of *S*. Enteritidis and *S*. Thompson across the cecal mucosa of freshly hatched chicks was visualized by electron microscopy (Popiel and Turnbull 1985). The uptake of *Salmonella* by macrophages was observed in the cecal lumen; the macrophages then became abnormal in appearance and were often ruptured, releasing organisms back into the lumen. Epithelial cell death was related to large numbers of bacteria. Bacteria were never observed in large numbers below the basement membrane, and there was no significant pathology in the lamina propria tissue. Wandering cells, identified as macrophages appeared to contain bacteria and were observed spanning the epithelial and lamina propria regions through breaks in the basement membrane. It is suggested that the passage of bacteria from the epithelium to the lamina propria is primarily the result of capture and transport within host macrophages.

The invasiveness of various serovars of *Salmonella* was directly compared in two studies in chickens. In ligated jejunal loops, zoonotic serovars of *Salmonella* were more invasive than host-specific serovars. Invasiveness of various serovars was more comprehensively assessed following oral inoculation of 1-week-old chicks in addition to inoculation onto cecal tonsils and into ligated jejunal loops. *S*. Typhimurium was found to be more invasive than *S*. Gallinarum at all sites tested, demonstrating that primary invasion does not correlate with systemic pathogenesis in chickens (Chadfield et al. 2003). Infection with *S*. Typhi-

murium is typified by acute enteropathogenic responses characterized by expression of CXC chemokines and a polymorphonuclear neutrophils (PMN) influx (Withanage et al. 2004) followed by massive multiplication in spleen and liver with septicemia.

Mortality rates, which vary from less than 10% to more than 80%, probably result from a combination of toxicity and dehydration. Strains of *S.* Enteritidis, in addition to being highly virulent for young chicks, can cause asymptomatic and chronic infections in older birds including commercial layers and broiler breeders. Infection soon after hatching can result in infection persisting until birds come into lay with the consequential vertical transmission. The extent to which egg contamination is a result of systemic bacteria reaching ovules, or a result of contamination, either in the oviduct or cloacae after egg formation and the extent to which this is a characteristic unique to *S.* Enteritidis, remain unclear. A study of orally infected adult birds showed that *S.* Enteritidis and *S.* Typhimurium strains are similarly able to colonize both the reproductive tract and any eggs that are forming in the oviduct prior to oviposition. Unfortunately, *S.* Enteritidis PT4 strains were not included in the study. More recently, in a comparison of six serovars injected intravenously into adult birds, *S.* Enteritidis PT4 was shown to colonize the reproductive organs of mature laying hens most efficiently (Okamura et al. 2001), which may well explain the association of this serovar with infected eggs. The inner shell and the shell membranes are frequently the main site of infection (Humphrey et al. 1991; Bichler et al. 1996) and egg infection may continue after fecal excretion ceases, suggesting that areas of the lower reproductive tract (isthmus and uterus) are the main sites of colonization.

Earlier work suggested that most *S.* Enteritidis egg infections arise from fecal contamination (Barrow and Lovell 1991). In contrast, *S.* Enteritidis colonization of the preovulatory follicles and ability to attach to ovarian granulosa cells were demonstrated, suggesting infection of eggs may occur as a result of systemic infection (Thiagarajan et al. 1994). More recent work suggests that *S.* Enteritidis may be able to colonize the reproductive tract by virtue of its ability to bind to secretions within the isthmus using type 1 fimbriae (DeBuck et al. 2003). However, all this latter work was carried out using *in vitro* assays.

COLONIZATION OF THE INTESTINE

The pattern of intestinal colonization by *S. enterica* serovars depends on the serovar itself, the nature of the infection and a number of host factors. Thus, for the typhoid-producing serovars, colonization is the initial stage of the disease process and not only are attachment, invasion, and intracellular survival important but also presumably so is survival in the intestine for long enough for these additional events to take place and to be shed from the intestine in the later stages of the disease (Rychlik et al. 1998). That virulence and colonization are integrally linked in these serovars is indicated by the fact that in a wide variety of attenuated mutants, clinical disease does not occur unless colonization takes place. However, a large number of serovars do not normally produce clinical disease in most food animals but colonize the intestine and are shed in the feces for several weeks. How far association with the mucosa is involved here remains to be seen.

Following early studies in the 1970s on the role of adhesion in colonization of the intestine by pathogens such as enterotoxigenic *Escherichia coli*, it was suggested that adhesion directly to the mucosa was an important component in colonization of the chicken ceca by *Salmonella*. However, there is little ecological rationale for a requirement for adhesion to the mucosa in an organ where the flow rate of contents is very low (poultry ceca empty two to four times a day), and there is no microscopical evidence for this. There is evidence for association with the mucosa in much lower numbers than expected, and this seems to reflect an invasive process (Barrow and Lovell 1988). The suggestion that colonization of the ceca/cecum and colon in different animal species is primarily a metabolic function of the bacteria may, however, be over simplistic. This is supported by the numbers of genes which contribute to colonization which are normally associated with adhesion or invasion (see below).

Host factors affect both the numbers of bacteria and the site of colonization. This includes the anatomy of different species which may present unique niches for colonization. In addition, the anatomy of the gut can change with increasing age with, for example, the development of the rumen in adult cows from the more simplistic preruminant calf. Nonspecific factors include the reduced flow rate of chyle in the cecum (ceca) and colon which

leads to increased bacterial growth and multiplication. The presence of a gut flora can also inhibit colonization by a number of pathogenic bacteria, including *Salmonella*. Its significance is indicated by the fact that newly hatched chickens, which have a very simple gut flora, are readily colonized by *E. coli* K12 and *S*. Choleraesuis, neither of which colonizes the adult gut (Barrow and Lovell 1988). Other host factors that control colonization include host genetic background. Recent studies have shown that the host genetic background can exert considerable influences on the extent of intestinal colonization and fecal shedding (Barrow et al. 2003). This effect is not related to immune responsiveness and is expressed within hours of infection but the mechanism is unknown.

Considerable variation occurs between colonization ability in the two major *S. enterica* disease-producing groups (pathovars). Colonization, generally defined as persistent isolation from the intestine of large numbers of bacteria, is an integral stage of the infection and disease process in both major pathotypes of *S. enterica*. Thus, there is considerable microbiological evidence that the typhoid-producing serovars, including *S*. Typhi (man), *S*. Gallinarum and *S*. Pullorum (birds), *S*. Dublin, *S*. Enteritidis, and *S*. Typhimurium (mice), and *S*. Choleraesuis (several mammalian species), are poor colonizers. After human infection by the oral route, *S*. Typhi disappears from the intestine until clinical disease occurs when it reappears in the gut and is shed in the feces. This also occurs with *S*. Gallinarum in chickens. In the chicken, most of the inoculum is destroyed in the stomach (gizzard) as a result of low pH, with just a minority required to produce invasion in the intestine. Thus, the oral LD_{50} for *S*. Gallinarum in chickens is 10^4 CFU, whereas it is less than 10 bacteria by any parenteral route, indicating that it is only necessary for a small number of orally inoculated bacteria to reach and penetrate the intestinal tissues.

Serovars of pathogenesis (pathovar) group 2 which typically do not produce typhoid-like infections in adult animals are able to colonize the intestine of several host species. As a result of this, they enter the food chain and are associated with human gastroenteritis. Whether host specificity occurs in this type of infection remains to be seen, but it is thought not since the colonization characteristic of strains of *S*. Typhimurium, *S*. Infantis, and typhoid serovars in mice and chickens are very similar (Barrow et al. 1994). One of the problems with studying disease-free colonization is that it is poorly understood in terms of localization of the bacteria in the intestine. A small number of studies with *E. coli* in mice (Poulsen et al. 1995) and unpublished studies using *Salmonella* in chickens (P. Adams et al., unpublished) suggest that most bacterial growth takes place at the interface between gut contents and mucosa where nutrients and electron acceptor concentrations are likely to be higher. A physical association with the mucosa can be detected microbiologically (Barrow and Lovell 1988) or by microscopy (P. Barrow and S. Hulme, unpublished), but the significance of this remains to be determined.

The ability to colonize the intestine is shared unequally by *Salmonella* serovars. The identification of microbial determinants has used two criteria for colonization: (1) isolation of the pathogen from the mucosa and (2) isolation from the feces as an indication of shedding. Although *a priori* it may be thought that (1) would reflect the initial stages of systemic infection/gastroenteritis and (2) may reflect a number of stages in the infection process, colonization is obviously a complex aspect of virulence, and the significance of association with the mucosa remains to be determined and may yet also be an integral aspect of disease-free colonization in animals and man.

The identification of colonization determinants in *S. enterica* has followed two routes: namely, the *in vivo* screening of randomly created transposon insertion mutants and the characterization of strains with defined mutations in genes thought to be of interest. Recently, studies have also been started using *in vivo* transcriptional analysis by microarray. All these studies have led to a change in our understanding and perception of colonization, and they may now be informed by the comparison of genome sequences from serovars that colonize with those that do not.

Studies where serovars are compared either by their genome sequence or biology must be interpreted with care since it is important to ensure that like is compared with like in terms of the disease produced by the serovars under study. Thus, comparisons of Typhimurium or Dublin with Gallinarum for colonization ability may be complicated by other differences in host specificity and virulence (and vice versa) (Carnell et al. 2007). However, comparisons between taxonomically closely related serovars, such as the group D serovars Dublin, Enteritidis,

Gallinarum, and Pullorum, are of great potential value. The genome analysis of *S*. Enteritidis and *S*. Gallinarum (Thomson et al. 2008) revealed that *S*. Gallinarum has a genome of slightly reduced size with a greatly increased number of pseudogenes (309 in comparison with 113 for Enteritidis and 25 for Typhimurium); *S*. Typhi has 204. These may be the result of gradual host adaptation as has occurred with *Mycobacterium leprae*, where reduced genome size and gene complement are apparent, or it may be more a result of loss of colonization ability incorporating poor external survival ability. Thus, *S*. Gallinarum has lost several catabolic pathways, which are likely to narrow the spectrum of substrates available for use as carbon and energy sources. These include a periplasmic alpha-amylase (*malS*) required for growth on long-chain maltodextrins, genes affecting growth on D-glutarate, and a mutation in the *hyaF* (hydrogenase I). In addition, *S*. Gallinarum 287/91 has several mutations in three operons required for the catabolism of 1,2-propanediol: *ttr*, *cbi*, and *pdu* operons encoding respiration using tetrathionate as an electron donor (*ttr*), cobalamin (vitamin B_{12}, *cbi*) biosynthesis, and 1,2-propanediol degradation (*pdu*), as has *S*. Typhi CT18, also regarded as a noncolonizer. Unpublished work suggests that combined mutations of *ttr* and *pdu* reduce colonization of *S*. Typhimurium in chicken (P. A. Barrow et al., unpublished). In addition to the anaerobic utilization of tetrathionate as a terminal electron acceptor, *S*. Typhi CT18 with mutations in *dmsAB* is unable to utilize dimethyl sulfoxide (DMSO). Although *S*. Gallinarum and *S*. Pullorum do not produce glycogen and are nonmotile, this is not a feature of noncolonizing mammalian serovars such as *S*. Choleraesuis and Typhi. *S*. Gallinarum 287/91 also possesses pseudogenes in several amino acid pathways including arginine degradation and ornithine decarboxylation. *S*. Choleraesuis also has a repertoire of pseudogenes more closely resembling *S*. Typhi, with mutations in genes encoding energy generation, carbohydrate, and amino acid transport (Chiu et al. 2005). Although many of these genomic differences are indicative of traits which may be important in intestinal colonization, in most cases no firm link has yet been made.

In these three serovars, pseudogenes occur in SPI-3, known to encode genes which affect colonization including *shdA*, *ratA* (Gallinarum), *sugR* and *rhuM* (Choleraesuis), and *cigR*, *marT* and *misL* (Typhi). Mutations in *shdA* and *misL* have been shown to reduce the colonization ability of *S*. Typhimurium in mice (Kingsley et al. 2000) and chickens (Morgan et al. 2004). A study with *S*. Typhimurium in mice (Kingsley et al. 2003; Dorsey et al. 2005) found that the *misL* gene product contributed to colonization and bound to fibronectin. The *shdA* gene affected colonization of both the cecum and Peyer's patch, whereas *ratA* contributed to cecal colonization only and *sivH* to Peyer's patch colonization. All except *sivH* affected shedding in the feces.

Given that bacterial fimbriae facilitate adhesion to a variety of surfaces and that *S*. Enteritidis, for example, has 13 fimbrial loci, it seems likely that at least some of these may be involved in colonization, perhaps through a physical association with the mucosa. The complement of fimbrial loci possessed by colonizing (*S*. Typhimurium and *S*. Enteritidis) and noncolonizing serovars (*S*. Gallinarum, *S*. Typhi, and *S*. Choleraesuis) suggests that the *sti*, *fim*, and *lpf*, and possibly *stb* and *sth*, may encode candidates for colonization determinants (Clayton et al. 2008). Mutations in *stiA*, *lpf*, and *fimA* had little effect on colonization, whereas a deletion in *pegA* (functional in Enteritidis but not in Gallinarum and replaced in the other serovars by the *stc* locus) produced a significant reduction in colonization in chicken. Screening signature-tagged mutants of *S*. Typhimurium indicated that SPI-6-encoded *saf* fimbriae may be involved in ileal colonization in pigs (Carnell et al. 2007), whereas the *stbC*, *csgD*, *pef*, and *sthB* fimbrial genes may have been involved in colonization of the avian gut (Morgan et al. 2004). Other studies using single mutations have not been able to implicate *fimA*, *csgA*, or *sefA* in chicken gut colonization (Thorns et al. 1996; Rajashekara et al. 2000), while *fimA* mutants showed short-term reductions in colonization in rats (Naughton et al. 2001). Although the *std* locus is found in all four serovars, Chessa et al. (2009) found that it contributed to colonization through binding to α(1,2)fucose residues in the murine cecum.

Mutation studies have indicated a role for stress proteins and lipopolysaccharide (LPS) (Turner et al. 1998) and a wider variety of other genes in colonization of chicken and calf when gut contents were combined with intestinal mucosal tissue (Morgan et al. 2004). The role of LPS in adherence and intestinal colonization may be explained by its involvement in cell envelope stability and associated resistance to bile salts, cell surface hydrophobicity, and the correct insertion and folding of

membrane proteins. The importance of other cell surface polysaccharides is suggested as mutants carrying insertions in colanic acid biosynthesis (*wcaE*) and enterobacterial common antigen biosynthesis (*wecE)* were attenuated in both chicken and mammalian intestinal colonization models (Morgan et al. 2004).

These studies highlight the importance of comparable samples for analysis. In this case, calf samples were homogenized calf ileal mucosa (including Peyer's patch) and chicken samples were whole homogenized ceca where the majority flora would have been isolated from the lumen. Thus, SPI-1, SPI-2, and SPI-4 genes contributed to colonization of the calf but not the chicken. SPI-3 genes were important in both host species. In this study, bacterial physiological traits were more important in colonization of the chicken than the calf. Thus, in addition to heat shock, uptake of L-serine, threonine, arginine, orotate, and hydroxyacetate were important. The *relA* gene was important, suggesting that amino acid starvation may be a factor. In addition to response to heat shock via *clpB*, transcriptional regulators such as *dks*, *hns*, and *kdgR* appeared to be important in both host species. Genes present in the Gifsy-1 and Gifsy-2 prophages were also important in the chicken but not the calf. A number of unusual genes such as the gene for diguanylate cyclase were also important to both host species, but the significance is unknown (Morgan et al. 2004). Gifsy-1 phage elements were also thought to be implicated in a subtractive hybridization study between *S*. Dublin and *S*. Gallinarum (Pullinger et al. 2008).

Very recent unpublished studies (P. Adams et al., unpublished) analyzing gene transcription by whole genome microarray in *S*. Typhimurium organisms harvested directly from the intestine of very young chickens suggest that the majority of the bacteria in the lumen contents show very little bacterial growth, although characteristic patterns of gene expression can be observed in comparison with bacteria harvested from the mucosa, including up-regulation of a number of fimbrial genes and operons associated with propanediol utilization.

The identification of colonization determinants not only increases our understanding of the basic infection biology of some of these closely related but functionally different set of bacterial pathogens. The data may also be used to identify antigens which might be used for vaccination, alone or expressed by a vector, or may be inactivated in a live vaccine such that the vaccine would be shed in the feces less than a wild-type strain and may therefore be more acceptable as a live, attenuated vaccine for use against a foodborne pathogen to reduce the risk of entry into the human food chain.

SALMONELLA INVASION AND ENTEROPATHOGENESIS

The various stages of pathogenesis of enteritis due to *Salmonella* are illustrated in fig. 14.1.

Introduction to Intestinal Barrier

Intestinal colonization of *Salmonella* is central to entry into the human food chain either through carcass contamination or preceding systemic infection and subsequent egg contamination. Acquisition by hosts of *Salmonella* can occur by both oral infection and aerosol infection through the respiratory system. After oral acquisition by the host, *Salmonella* pass through the stomach and enter the intestinal tract. It is here that it first interacts with the mucosa and this interaction will dictate the outcome of infection. The mucosal surface of the gastrointestinal tract is lined by epithelial cells sealed by tight junctions. This forms a barrier to both macromolecules and microorganisms, and cellular and physical integrity are essential for correct intestinal function. Within the intestine, the specialized follicle-associated epithelium (FAE) contains antigen supporting M cells in addition to enterocytes. The M cells represent about 50% of the FAE, with variation between species. *Salmonella* can attach to and invade the enterocyte or be scavenged by patrolling mucosal phagocytes. The infection of enterocytes can have a number of effects including tissue thickening and necrosis, inflammatory response, and fluid secretion. The term "enteropathogenic response" has been coined to cover the combination of inflammatory and secretory responses often directly linked to diarrhea in a number of species. After attachment of *Salmonella* to the enterocytes, there is a characteristic remodeling of the membrane around the *Salmonella* leading to lamellipodal extension and envelopment of the bacterium (McCollister and Vazquez-Torres 2006). The internalized bacteria are maintained with a vacuole within the enterocyte, but eventually the bacteria may multiply and apoptosis-like cell death may ensue (Mastroeni 2005).

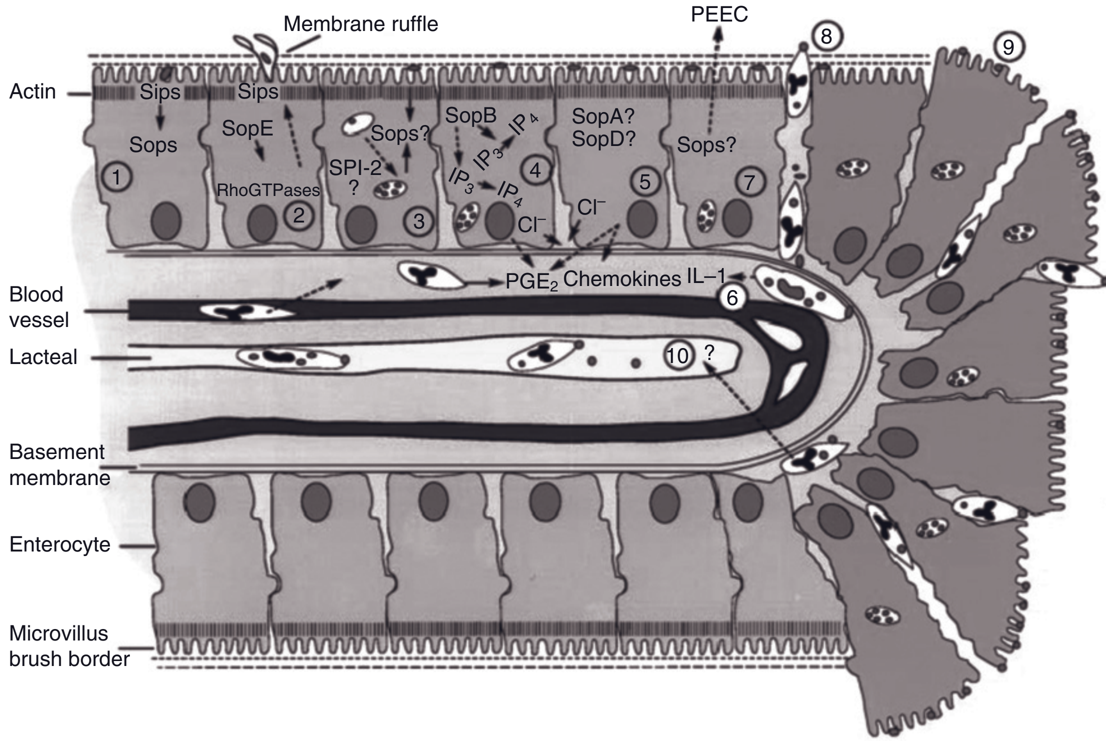

Figure 14.1. Schematic representation of stages of pathogenesis of enteritis. (1) Interaction with enterocytes, delivery of Sop proteins into cytoplasm via TTSS-1 and Sip-dependent pathway; (2) Sip proteins, SopE, and other Sops induce membrane ruffling inducing invasion; (3) bacteria in intracellular membrane-bound vesicles; (4) SopB affects inositol phosphate-related signaling events leading to electrolyte transport and fluid secretion; (5) infected epithelial cells secrete chemokines attracting inflammatory cells to foci of infection; (6) *Salmonella* interaction with inflammatory cells stimulating release of proinflammatory cytokines enhancing inflammation; (7) stimulation of trans-epithelial migration of granulocytes by *Salmonella*; (8) phagocytosis of *Salmonella* bacteria by inflammatory cells; (9) extrusion of infected enterocytes from villus surface leading to villus blunting and reduced fluid absorption; (10) migration of infected cells and bacteria to draining lymphatics carrying bacteria to systemic sites. PEEC, pathogen-elicited epithelial chemoattractant; PGE2, prostaglandin E2. (Figure used with permission). (See color plate)

Timing and Positioning of Salmonella *in the Intestine*

The timing and outcomes of infection are dependent on a number of factors that include host factors of age, genetics, immune status, and concurrent diseases. The age and health status of the animal may also affect the development of qualitative or quantitative changes in the intestinal microflora. The flow rate and lumenal environment through which the bacteria pass to enter the intestines differ between species and will have an impact on colonization.

Studies have looked at both natural infections via the oral route in a number of species and ligated intestinal loops. Inflammatory responses can be detected within 3–6 h of infection in experimental animals. These studies indicate the rapidity with which infection takes place in addition to the host response. More specific studies using calf intestinal ligated loops show that invasion can occur within minutes of infection (Frost et al. 1997), with a rapid host response involving diapedesis of neutrophils into the lumen of the intestine within 30 min of exposure. While this study provides a quantitative temporal estimate of the initial interaction with the host, the disrupted gut flora of the ligated loop may affect the timing and nature of the interactions between the bacteria and the host.

The localization of infection may be sporadic, and studies in poultry with green fluorescent protein (GFP)-tagged-*Salmonella* indicate the formation of intermittent sites of attachment and invasion in the cecal lymphoid tonsils (S. Hulme et al., unpublished).

Molecular Mechanisms of *Salmonella* Invasion

The initial interaction between pathogen and epithelial cell through an attachment process is still poorly understood. However, there is increasing information available from comparative genome studies (see colonization section). The role of the many fimbrial structures expressed by a number of *Salmonella* serovars remains to be fully elucidated, and studies indicating that individual fimbria contribute to but are not essential for colonization suggest a degree of redundancy and complex interactions in terms of *in vivo* expression profiles.

Invasion

A major advance in the understanding of the *Salmonella* infection process came with the identification of a number of *Salmonella* pathogenicity islands (SPIs) and other virulence genes associated with enhanced invasion and survival within both phagocytic and nonphagocytic cells. Pathogenicity islands are discrete inherited areas of the genome which vary in the numbers of genes they contain and in their role (see section on genome content below). Arguably, the two key SPIs are SPI-1 and SPI-2 which contain the TTSS systems TTSS-1 and TTSS-2, respectively. These two islands were initially reported as being involved in invasion (Galan and Curtiss 1989; Mills et al. 1995) and intracellular survival (see section below). However, other genes and islands often coding for TTSS effector proteins are also involved in invasion and intracellular survival. SPI-1, SopE2, and SPI-4 have all been shown to act in concert with SPI-1 for invasion (Mirold et al. 2001).

The TTSS's promote the transfer of proteins directly from bacteria in contact with infected host cells into the host cell cytoplasm where they have a range of effects (Hueck 1998; Ghosh 2004). The transferred proteins are referred to as effector proteins and a number of these have been characterized in a range of bacterial species (Galan 2007). TTSS-1 is activated on contact with host cells and is involved in the secretion of at least 12 effectors which are injected into host cells. SP-1 is about 40 kb in size, has a lower G + C content (47%) in comparison with the rest of the genome, and is composed of two main elements. The SPI-1 TTSS is encoded by ca. 30 genes whose products form needlelike surface appendages that mediate the delivery of effector proteins into the host cell. A small second element *sitABCD* at a flank of SPI-1 encodes an iron uptake system not thought to be related to invasion and enteritis. SPI-2 promotes invasion in a number of *Salmonella* serotypes including Gallinarum, Typhimurium, Pullorum, and Enteritidis and is also present in other serotypes which do not generally produce systemic disease but are associated with gastroenteritis, such as Hadar and Infantis (www.sanger.ac.uk/projects/microbes; Ochman and Groisman 1996).

While SPIs play a key role in infection, other critical factors are involved in disease. The virulence plasmids are vital for infection to proceed and are found in most serovars associated with systemic disease (see below). Although these are known to be important in the systemic phase of the disease, they are also, in a few cases, important in the initial colonization/attachment/invasion stage probably through the elaboration of fimbriae, which in the case of *S*. Gallinarum resemble the *E. coli* K88 (F4) fimbrial genes (Rychlik et al. 1998).

SPI-1 and Invasion

TTSS-1 is considered responsible for mediating the intestinal invasive phase of *Salmonella* infection. It is induced under conditions similar to those found in the small intestine such as late-log phase growth in rich media containing high levels of salt and increased temperature, conditions that are thought to replicate entry into the environment of the small intestine. Invasion is mediated by at least five effector proteins which are involved in major cytoskeleton remodeling, the genes for which are found outside SPI-1. The effector proteins are SopE/E2, which are involved in membrane ruffling and initial promotion of entry involving bacterial envelopment (Hardt et al. 1998a; Bakshi et al. 2000; Stender et al. 2000), SopB, which modifies phosphoinositide metabolism and indirectly activates Rho GTPase leading to micropinocytosis (Norris et al. 1998; Zhou et al. 2001; Terebiznik et al. 2002; Hernandez et al. 2004), and SipC, which inserts into the host cell membrane and promotes actin nucleation with a cooperative effect from the effector SipA,

which, along with SipC, potentiates actin bundling (Hayward and Koronakis 1999; Zhou et al. 1999; Scherer et al. 2000; Jepson et al. 2001; McGhie et al. 2001; Higashide et al. 2002; Lilic et al. 2003). SipB triggers the activation of intracellular caspase-1 within resident macrophages, which induces apoptosis in the macrophage facilitating escape of virulent bacteria. The effector SptC has an essential role in antagonizing the function of the SopE/E2 effectors, allowing for recovery of cellular architecture post invasion (Fu and Galan 1999; Stebbins and Galan 2000; Kubori and Galan 2003). The temporal coordination of these effectors is as important as their function because the guanosine exchange factor (for RhoGTPases) modification and mitogen-activated protein kinase pathway inhibition activities of SopE and SptP are opposing and require coordination to lead to transient membrane ruffling (Kubori and Galan 2003).

Besides the promotion of invasion, the TTSS-dependent effector proteins have a range of effects on the host cells and tissues. In the intestines, they are involved in the enteropathogenic response, stimulating increased secretion of fluid and attraction of phagocytes into the lumen and disrupting tight junction structure and function (Wallis and Galyov 2000; fig. 1). Sop B is an inositol phosphate phosphatase which induces a transient increase in concentration of inositol(1,4,5,6)phosphate which antagonizes the closure of chloride channels influencing net electrolyte transport and fluid secretion. The roles of SopA and SopD are not fully understood, but they also contribute to the enteritis phenotype (Jones et al. 1998). Many of the studies investigating their function *in vivo* have been carried out using the ligated loop model and so do not assess the effect of these on the whole disease process.

Knockouts of SopB and SopD, both of which are secreted by TTSS-1, reduced enteropathogenesis but did not affect invasion, indicating a dual role for effectors through this secretion system (Wallis et al. 1999). SopA, SopB SopD, SopE2, and SipA act in concert with each other to induce diarrhea in calf infections (Wallis et al. 1999).

TTSS-1 and its effectors also play a role in the interaction between *Salmonella* and phagocytic cells. Studies of macrophage invasion show that SPI-1 plays a role in directing cells away from the complement-mediated pathways of engulfment toward an SPI-1-mediated vacuolation with reduced antimicrobial activity (Drecktrah et al. 2006). The importance of the vacuole environment is shown by the differences in gene regulation between *Salmonella* after complement or IgG opsonization or SPI-1-promoted invasion. This suggests that *Salmonella* is able to deliver itself into a more receptive intracellular niche. Thus, TTSS-1 function is central in both directing invasion and enteropathogenic responses in the intestine.

The role of SPI-1 in systemic infection is less clear, and TTSS-1 mutants in typhoid-causing strains are still able to cause systemic infection of the mouse and chicken. There are also recent reports of *S.* Senftenberg isolates which lack SPI-1 causing enteric disease in humans (Hu et al. 2008). Mechanisms of invasion other than those mediated by SPI-1 must therefore exist. It has been proposed that M cells offer a route through the mucosal barrier which is independent of the SPI-1-mediated mechanisms (Martinez-Argudo and Jepson 2008).

Further dissection of the molecular processes involved in *Salmonella* infections is complicated by the number of stages that are involved in the infection process. Global approaches have been used to identify genes involved in the initial stage of infection in cattle where colonization is combined with attachment/invasion (Morgan et al. 2004). However, this approach may miss genes where there is redundancy in function and or species specificity, in which case gene expression studies may be a more obvious way forward.

Salmonella-directed invasion is achieved through the manipulation of the host cytoskeleton machinery. *In vivo*, there is a set sequence of invasion of both enterocytes and M cells that overlie the domed villi. The extent of both depend on the species involved with more enterocyte involvement in a species such as the calf, where enteritis is manifest, and mainly M cell involvement in the mouse where systemic disease rather than enteritis predominates. The *Salmonella* cells induce membrane ruffling within minutes of contact with either cell type, which results in their envelopment and uptake within membrane bound vesicles. *In vitro*, a reduction of internalization can be achieved by interfering with either actin using clathrin or microtubules using cytocholasin-D. Interestingly, these studies also show residual uptake, suggesting that other mechanisms may promote internalization of bacteria albeit at a low level (Green and Brown 2006). The primary mechanism of internalization appears

to be independent of host species as results looking at SPI-1 (internalisation) and SPI-2 (survival) are equivalent when mutants of these are tested on enterocytes and macrophages from the human, mouse, cow, pig, and chicken.

Cell Signaling

Specific proinflammatory markers have been identified in *Salmonella*. Lipid-A, the major immunostimulatory component of LPS, stimulates through TLR-4, and flagellin, produced by most *Salmonella* serotypes, is a strong agonist for TLR-5 in a number of host species including humans and poultry (Takeda et al. 2003; Iqbal et al. 2005). While some studies have indicated that invasion is vital for production of IL-8, live bacteria are not required to stimulate proinflammatory cells *in vitro*. However, the intestinal mucosa provides a barrier to prevent nonspecific stimulation of Toll-like receptors (TLRs). The importance of the different TLRs in different host species and the expression of antagonistic pathogen-associated molecular patterns (PAMPs) by the different *Salmonella* serovars may be relevant to the host response and to the ability to control or restrict bacteria. Studies of inflammatory responses during the early stages of poultry infection have shown induction of cytokine and chemokine response involving the production of proinflammatory cytokines IL-6 and IL-1 and members of the CXC and macrophage inflammatory proteins (MIP) chemokines (Kaiser et al. 2000; Withanage et al. 2004, 2005). However, generalization about the induction of immune responses cannot be made as there are differences between serovars in their ability to induce different responses (Kaiser et al. 2000; Chappell et al. 2009).

The mechanisms involved in the strain-to-strain differences may correlate with differences in the effector systems between *Salmonella* serotypes. A number of *Salmonella* effector proteins contribute to the downregulation of stimulatory pathways or expression of downregulatory pathways in host cells. The AvrA protein has been studied extensively and has been shown to reduce inflammation and to help maintain cell junction integrity between enterocytes (Liao et al. 2008). AvrA acts through direct interaction with beta catenin-activated signaling within the host cell which indirectly affects cell proliferation in the villus, thereby increasing dissemination from the gut. SpiC, found in SPI-2, has been shown to induce suppressors of cytokine signaling and IL-10 and prostaglandin E_2 production (Uchiya et al. 2004; Uchiya and Nikai 2005). It is not known if other specific effectors are involved in moderation of the immune function or whether gene regulatory differences as suggested by Streckel et al. (2004) or differences in gene complement yet to be defined are involved. Some differences may be due to direct modification of signaling pathways rather than differences in initial stimulation, and it has been noted that the avian specific serotypes Gallinarum and Pullorum both lack flagellin and are the major causative agents of typhoid in poultry. In this case, it has been suggested that TLR-5 is central to the avian immune response to bacterial pathogens and that these serovars lack flagella and therefore avoid this immune alarm system (Iqbal et al. 2005).

The TLRs are not the only receptors important in *Salmonella* infection. Terminal alpha(1,2)fucose residues in the cecal mucosa act as important mucosal receptors for Std fimbriae (Chessa et al. 2009). Little is known about host species variation in these receptors. There are a number of processes during infection where host cell receptors are required for attachment prior to invasion. Some receptors are also involved in host cell signaling and which will lead to changes in host cell biology. Thus, in some cases, the lack of interaction may be purely physical in its effects, while in others it may have major effects on host responses.

Role of Macrophages

A central observation during *Salmonella* infection in many species is the attraction of neutrophil granulocytes (heterophils in poultry) into the area of infection, usually the intestine. Infection of enterocytes leads to the induction of chemokines and other chemotactic signals including IL-8, K60 (a CXC chemokine), MIP 1β, and IL-1β (Withanage et al. 2004, 2005), leading to the recruitment of macrophages to the site of infection; the role of these signals in driving pathology is unclear. The current dogma is that inflammatory responses triggered by the infection process lead to inflammatory damage with associated pathology. However, studies with infection by an avirulent *S.* Infantis in highly susceptible gnotobiotic pigs induced neutrophilia in mucosal tissue but without tissue damage. This neutrophilic priming is protective of secondary infection with virulent *S.* Typhimurium, suggesting that other factors and the timing of the inflammatory response may be important in the development of

enteropathogenic infection (Foster et al. 2003). This protective phenomenon may have practical prophylactic value, and there are several earlier references to cross-protection between unrelated pathogens as a result of this effect (Mackaness 1964).

Host Differences

There are clear differences between host species in the way in which they react to infection by different serovars. Some differences must clearly be due to differences between hosts following observation of differences in pathology resulting from infection of different hosts by the same serovar.

Translocation

Either following enteritis or as a result of the initial intestinal invasion/uptake stage in systemic disease, bacteria enter the lamina propria prior to entering the lymphatic/circulatory system to reach the spleen, liver, and other major sites of multiplication. It has been proposed that *Salmonella* invasion of macrophages is important in dissemination of the bacteria in the host and that intracellular spread is mediated by bacteria internalized in macrophage cells. However, recent bovine infection studies with *S.* Dublin and *S.* Abortusovis suggest that initial dissemination may not rely fully on this intracellular dissemination and that the majority of bacteria post intestinal invasion may be found free in efferent intestinal lymphatics of cattle until they are removed from circulation by macrophage-like cells within the spleen and liver (Pullinger et al. 2007). These observations indicate the importance of microbial mechanisms to escape from phagocytic cells in the mucosa.

SYSTEMIC DISEASE

Introduction

Salmonella infection can be regarded as a paradigm of the infection biology for intracellular bacterial pathogens. Although intracellular multiplication is a major component in enteritis and systemic disease and may occur more extensively as animals become increasingly sick, it is a key stage in the infection process in the serovars that typically produce typhoid-like diseases in man and animals.

Although for the typhoid-like disease producing serovars the systemic phase of infection is central to explaining the infection biology and epidemiology, similar infections may also occur as a result of infection with the remaining serovars which generally do not produce systemic infection in normal, healthy adult animals. Thus, infection of very young animals such as chickens and pigs within hours of birth or hatching may result in a disease which closely resembles typhoid. This occurs more frequently when no antibody is present in the young animal either as a result of no uptake of colostrum or no maternal IgY in the case of birds. This syndrome is similar to the colisepticemia observed in young animals which are depleted of protective antibody. The majority of *S. enterica* serovars are also capable of inducing enteritis in a number of species of animals including man which may lead to debilitation and dehydration itself, reducing the capacity of the host to resist infection, leading to a progressive systemic disease and death.

Salmonella Serovars and Systemic Disease

The infection biology of the typhoid-producing serovars is similar for all infections. These serovars tend to produce disease in a small number of species of hosts and are referred to as host specific, host restricted, or host adapted. The hosts with which they are most frequently associated may not be the hosts where the most typical form of disease is found, and some anomalies occur which complicate the taxonomic picture. Thus *S.* Typhi and Paratyphi produce typical typhoid in man but little disease in other species, either naturally or experimentally. *S.* Gallinarum is associated with typhoid in poultry and birds. *S.* Dublin and *S.* Choleraesuis are associated with a systemic infection in cattle and pigs, respectively. However, experimentally, *S.* Dublin produces a more typical clinical typhoid in mice and *S.* Choleraesuis does so in many mammalian species. *S.* Abortusequi and *S.* Abortusovis, associated with systemic disease and involvement of the reproductive tract in equines and ovine and caprine species, respectively, also produce murine typhoid under experimental conditions. Interestingly, *S.* Typhimurium and *S.* Enteritidis, which are associated causally with the highest proportion of cases of human gastroenteritis and *Salmonella*-associated food poisoning, also produce typical typhoid in mice. Indeed, murine typhoid produced by *S.* Typhimurium infection has been the model of choice for studying pathogenesis for many years. Whether the susceptibility of the mouse is unique in comparison with other species remains to be seen.

Host Specificity

There is little evidence that host specificity occurs with gastroenteritis. Adult humans may be affected by a large number of serovars, whereas young animals may be affected by serovars which induce typhoid in the adult animal in addition to *S.* Typhimurium and one or two other serovars. Adults which are immunologically compromised in some way, for example, hormonally (e.g., as a result of pregnancy), may also be susceptible to enteritis.

However, the basis of host specificity in typhoid-like infections is very poorly understood (see above). It has been suggested that the ability of specific serovars to persist within particular host macrophages may correlate with serovar-host specificity. For example, a comparison of the uptake and persistence of serovar Typhi in human and murine macrophages correlates with the virulence of this serovar in humans but not in mice (Ishibashi and Arai 1990; Alpuche-Aranda et al. 1995) Furthermore, *S.* Typhimurium persists in higher numbers than *S.* Typhi in primary murine macrophages *in vitro* (Vladoianu et al. 1990). Similar results have been found with *S.* Typhimurium and *S.* Gallinarum in murine and avian macrophages (P. Wigley, unpublished). However, in contrast, there was no correlation between the virulence of different *Salmonella* serovars for cattle and pigs and factors such as bacterial uptake, intracellular persistence, and killing or production of cytokines in bovine or porcine macrophages *in vitro* (Watson et al. 2000).

The role of *Salmonella*/macrophage interactions in determining host range and the severity of systemic disease remains unclear. It is clear that serovars of differing specificities may have unique pathogenicity islands which may be associated with this characteristic. Thus, genes in SPI-1 and SPI-2, which may have evolved differently in different serovars, may be implicated. These include genes that encode translocon proteins (SipD, SseC, and SseD) of both SPIs 1- and 2-encoded TTSS, *sptP*, which encodes an effector protein that inhibits the mitogen-activated protein kinase pathway of the host cell, and genes which encode effector proteins (SseF and SifA) that are important in placing the *Salmonella*-containing vacuole in a juxtanuclear position (Eswarappa et al. 2008). In addition, fimbrial genes and function may contribute (Clayton et al. 2008; Thomson et al. 2008; Guo et al. 2009).

Evolutionary Value of Systemic Disease

It is understandable that *Salmonella* strains that induce enteritis do so in order to increase bacterial multiplication in the intestine followed by induction of increased fluid secretion with the associated shedding into the environment of large numbers of bacteria which are likely to be disseminated more widely than if they were excreted in more solid feces. Since most *Salmonella* serovars produce no disease in birds and are readily isolated from reptiles, it may be argued that these animals are their natural hosts in evolutionary terms and that infection of mammals could be regarded as disadvantageous, with the result that the pathogen induces enteritis to ensure rapid and widespread dissemination from the mammal to increase the chance of infection of a bird or reptile.

Those serovars that induce typhoid-like infections have become adapted to their particular host species or groups of closely related species. Adaptation possibly occurred in an environment where there were small numbers of individual animals or groups of animals that may have had little contact with each other. This is certainly the case for the progenitor of the domestic fowl, the jungle fowl.

Using human typhoid and avian typhoid (*S.* Gallinarum and *S.* Pullorum) as models, these organisms have adapted to survival and multiplication within macrophages and macrophage-like cells within the body. They colonize the gut poorly, and the ID_{50} for *S.* Gallinarum in chickens is ca. 10,000 organisms by the oral route. In contrast, the numbers in the spleen in the later stages of disease may reach log 10^6 to 10^7 per gram of tissue. At this stage, the bacteria break out from these organs, enter the blood, and form areas of necrosis, possibly around lymphoid tissue in the gut from whence they are shed from the intestine in semiliquid feces. However, they do not survive well in the environment so that this form of shedding may be relatively short term.

In addition, several of these serovars have adapted to localization in the reproductive tract with resulting abortion, with the production of heavily infected animals at birth or transfer to eggs with the production of heavily infected but live progeny. This strategy avoids the rather hit-and-miss approach of infection associated with intestinal shedding in relatively small numbers and where contact between individuals may be infrequent. It is also associated with a degree of attenuation; *S.* Pullorum, which is

taxonomically closely related to *S*. Gallinarum, is less virulent but demonstrated vertical transmission at a much greater frequency.

Another strategy adopted by *S*. Typhi is persistent infection involving the gall bladder, which results in long-term shedding by convalescent animals. The genes associated with these characteristics are totally unknown but are thought to involve genes in SPI-2, 3, or 4.

These observations demonstrate the application of different strategies that a large taxon with different infection biologies adopts to ensure dissemination between individuals and the ways that the organism copes with the associated constraints.

Multiplication in the Tissues

The mechanisms of survival and persistence of *Salmonella* within the liver and spleen of an infected host have received a great deal of study, particularly in the murine model of infection. At present, comparatively little is known about the specific niches in which bacterial replication takes place. Results from detailed histological and microscopic studies suggest that serovar Typhimurium is likely to reside within murine splenic or hepatic PMNs, hepatocytes, and/or Kupffer cells during the early stages of disease and within macrophages during the later stages of the infectious process (McCollister and Vazquez-Torres 2006).

Interactions with mononuclear phagocytes, predominantly macrophages, are central to the pathogenesis of systemic salmonellosis. The ability to survive within macrophages is important both in the early phase of infection before the acute inflammatory process begins and later during immunity when macrophages have been activated and a granuloma has formed.

A recent study using multicolor fluorescence microscopy to visualize individual serovar Typhimurium bacteria within the livers and spleens of mice enabled the study of interactions between different bacterial populations within the same animal (Sheppard et al. 2003). The results demonstrated that an increase in bacterial load within an organ could be attributed to the establishment of new foci of infection, rather than increased numbers of bacteria per phagocyte. This suggests that *Salmonella* are not replicating freely within phagocytes in systemic tissues and highlights how little we know about the very fundamental aspects of *Salmonella* pathogenesis.

A number of antibacterial components, including the products of the loci for TLR-4, Nramp1, NADPH oxidase, and IFNγ, are important in resistance to infection, and the ability of the bacterium to combat these antibacterial effects is important in survival and persistence.

Interaction between microbial surface ligands with macrophage receptors results in internalization into a membrane-bound phagosome. To avoid maturation of the phagosome, which would result in increased microbial killing, *Salmonella* are able to inhibit fusion of the phagosome with lysosomes through the activity of SPI-2 gene functions (Uchiya et al. 1999). Phagosomes containing *S. enterica* avoid interactions with NADPH oxidase- and inducible nitric oxide synthase (iNOS)-containing vesicles (Chakravortty et al. 2002), whereas they may acquire lysosomal-associated membrane glycoproteins (Rathman et al. 1997), suggesting the ability to acquire biomolecules in transit from the exocytic pathway, presumably for nutritional purposes.

Although phagosomes acidify their contents to a pH of less than 5, *Salmonella* are able to slow the rate of acidification (Alpuche-Aranda et al. 1992). Acidification activates transcription and secretion of SPI-2 effectors. Transcriptional profiling of *S*. Typhimurium in murine and avian macrophages using microrrays has indicated that phagosomes are also low in phosphate, magnesium, and manganese, with the bacteria utilizing carbon sources such as gluconate and 2-propanediol (Eriksson et al. 2003; Adams et al., unpublished). There is some Fe depletion, which is also a result of Nramp1 (Slc11a1) activity as a metal ion symporter. Reduced divalent cation concentration also up-regulates SPI-2 expression (Zaharik et al. 2002).

The major macrophage killing mechanisms include NADPH oxidase, nitric oxide, and cationic antimicrobial peptides (defensins). The importance of NADPH oxidase is shown by the susceptibility to salmonellosis of animals suffering from chronic granulomatous disease in which this activity is lacking and no respiratory burst occurs as a result of phagocyte infection. SPI-2 effectors inhibit the fusion of the enzymatic components with the phagosome, enhancing bacterial survival. The generation of superoxide is highly toxic to *Salmonella*, largely through its activity on the Fe-S clusters of respiratory enzymes. The small amount that persists may be inactivated by the periplasmic Cu/Zn superoxide dismutase (SOD) encoded by the Gifsy-2 prophage

and the *sspJ* locus within SPI-2 (Tsolis et al. 1995; De Groote et al. 1997; van der Straaten et al. 2001). Nitric oxide and its oxidized derivatives, N_2O_3 and NO_2 and peroxynitrite, appear to be associated with the later stages of infection (Vazquez-Torres et al. 2000) and are induced by LPS/TLR-4 and IFNγ signaling cascades. Peroxynitrite is highly antibacterial but is excluded from the *Salmonella*-containing phagosome by the same inhibition mechanism that prevents fusion with the NADPH-containing vesicles that also generate iNOS (Vazquez-Torres and Fang 2001).

It is clear that TTSS-2 encoded on SPI-2 is pivotal in influencing systemic disease in mice. Whereas TTSS-1 is expressed by extracellular bacteria, TTSS-2 is expressed by intracellular *Salmonella*, and TTSS-2-dependent secreted effectors are important in modulating vesicular trafficking inside *Salmonella*-infected cells. It is becoming increasingly clear that SPI-2 is a major virulence factor during infection of food-producing animals including cattle and poultry.

SPI-2, Other SPIs, and Virulence

Pathogenicity islands are clusters of genes on the chromosome with a related function that show different degrees of stability, and are suggested to have been acquired horizontally. They are frequently inserted at genes encoding tRNAs, which themselves are often integration sites for bacteriophages, one of the driving forces in microbial evolution. More than 10 SPIs have been found in *S. enterica* and all have genes which are associated with virulence functions.

Although SPI-1 is primarily associated with the invasive phase, it does also have functions relevant to macrophages and dendritic cells (DC), at least in the early gut-associated phases of infection (Galan 2001). Thus, during invasion of the gut, the SPI-1-encoded SipB protein triggers activation of intracellular caspase-1 within resident macrophages. Caspase-1 induces apoptosis in infected macrophages resulting in microbial escape. There is evidence for bacterial translocation from the intestine to mesenteric lymph nodes and spleen in intracellular and extracellular phases.

SPI-2 is vital for intracellular survival and multiplication within the tissues (spleen and liver) and is associated with the ability to multiply within intracellular vacuoles. SPI-2 is present in *S. enterica* but not in the phylogenetically older *Salmonella bongori*, and has thus been acquired more recently than the division of these two species. It is sometimes thought to be an association with the ability to produce disease in warm-blooded animals, although the pathogenesis in reptiles has not been determined in detail. SPI-2 appears to be relatively stable since no genes associated with DNA transfer or mobility are detectable. In addition, effector protein genes associated with SPI-2 may be found outside the locus. SPI-2 is 40 kb in size, is inserted next to the tRNA gene *valV*, and is composed of two major elements. A 25-kb portion with a low G + C content of 43% and which is only found in *S. enterica* is essential for systemic virulence. This contains genes for a TTSS which is separate from that encoded by SPI-1 and which is activated intracellularly rather than under the conditions found in the intestine. Another element with a G + C content of 54% is present in SPI-2 in both *Salmonella* species and contains genes encoding a tetrathionate reductase, which have been found in other bacterial genera.

All the components of this TTSS, several effector proteins, and the two-component regulatory system (SSrAB) are encoded by SPI-2. Mutations in SPI-2 or SsrAB attenuate *Salmonella* strains for their ability to multiply intracellularly (Shea et al. 1999). As mentioned above, SPI-2 encodes the ability to prevent fusion of phagocytic oxidase with the phagosome, and thereby prevents the antimicrobial effects of reactive oxygen and nitrogen species. Mutants defective in SifA function are unable to maintain the *Salmonella*-containing vacuole (Beuzon et al. 2000). A delayed caspase-1-dependent form of apoptosis is associated with SPI-2 function (Monack et al. 2001). The presence of tetrathionate reductase genes (three structural genes plus the *ttrSR* two-component system) are important in virulence and indicate that redox conditions within the phagosome are likely to be low, as also indicated by studies in which mutations in genes encoding components of NADH dehydrogenase II and cytochrome d oxidase (as opposed to NADH dh1 and cytochrome o oxidase) are also attenuating (Turner et al. 2003).

Genes within and outside the SPI-2 locus have been identified as encoding effector proteins. The three proteins SseBCD function as translocators of effector proteins including SSeFG, located within SPI-2. SpiC, thought to be a component of the TTSS, also functions in interference with

intracellular vacuolar traffic. Outside SPI-2, several translocated effector proteins have been identified, encoded by genes scattered around the chromosome, including SifA, SifB, SspH1, SspH2, SlrP, and SseI which share structural similarities in their N-terminal domain, and also PipB, encoded by SPI-5, PipB2, and SopD2. Some of the effector proteins outside SPI-2 are encoded by bacteriophage elements, indicating that evolution is taking place not within the stable SPI-2 locus but as a result of accumulation of genes or mutations in genes outside SPI-2.

Although SPI-2 null mutations and deletions induce profound attenuation in systemic virulence (Jones et al. 2007), mutations of individual genes do not confer the same level of attenuation. SifA appears to be important for accumulation of endosomal components in infected cells and maintains the integrity of the phagosomal membrane during infection (Beuzon et al. 2000).

The SsrAB regulatory element is modulated by the OmpR/EnvZ two-component regulatory system responsive to osmolarity. The PhoPQ two-component system is also thought to regulate SPI-2 gene expression (Cirillo et al. 1998; Deiwick et al. 1999). It is thought that the environmental elements that contribute to the conditions within the infected phagosome contribute to inducing gene expression within SPI-2. These include low Mg2+ and P– concentration (Deiwick et al. 1999). Although acidification occurs within the phagosome, it is thought that this induces assembly of the TTSS rather than expression of its genes. SsrAB does not control *ttr* gene expression but is controlled by its own regulatory element, TtrRS (Hensel et al. 1999).

A smaller SPI of 17 kb, SPI-3, encodes a number of genes which contribute to intracellular survival involving resistance to antimicrobial cellular mechanisms and the ability to cope nutritionally with the poor nutritional conditions found in the phagosome, including Mg2+, amino acids, purines, and pyrimidines. The high-affinity Mg2+ uptake system encoded by the MgtCB system is important for survival and multiplication within macrophages (Blanc-Potard and Groisman 1997). The proteins are located in the cytoplasmic membrane. A number of genes in SPI-3, including *sugR* and *misL*, may be absent in some serovars such as *S*. Choleraesuis, which typically produce systemic disease. How far these differences are related to host specificity remains to be determined.

SPI-4 (23 kb) has a G + C content of 45% and is also located near a tRNA element but it has been poorly studied. There is some evidence for an association with intestinal colonization in calves but not chickens, which may reflect the nature of samples (mucosal scrapings) taken in this study (Morgan et al. 2004). However, it does also suggest that colonization may require intracellular survival/ multiplication. A more recent study has shown that SiiCDF form a type 1 secretion system for the secretion of SiiE, a large protein (600 kDa) which is involved in the bovine intestinal colonization. There was less attenuation in *siiE* mutants of *S*. Enteritidis than of *S*. Typhimurium in studies in mice (Kiss et al. 2007).

SPI-5 is largely associated with the ability to produce enteritis, but one gene, *pipB*, encodes an effector protein translocated by SPI-2 which contributes at least to survival within murine macrophages (Pfeiffer et al. 1999) and avian epithelial cells and macrophage-like cells (Li et al. 2009).

SPI-6 contains the fimbrial *saf* locus which is thought to contribute to colonization (Clayton et al. 2008) and uncharacterized genes that promote intracellular survival within macrophages (Klumpp and Fuchs 2007).

The remaining SPIs have been less well characterized, although uncharacterized genes in SPI-7, 8, and 10 in *S*. Typhi contribute to intracellular survival in human macrophages (Faucher et al. 2005). Apart from SPI-7 (133 kb), they are all considerably smaller (7–33 kb). SPI-7 is restricted to *S*. Typhi and encodes the production of the Vi antigen and a type IVB pilus. Neither SPI-9 nor SPI-10 genes have been characterized in terms of virulence.

Many *Salmonella* serovars contain autonomously relicating plasmids. Most serovars that typically produce systemic disease contain large (60–100 kb) plasmids which are generally not conjugative but which are essential for the expression of virulence (Gulig et al. 1993). The exception is *S*. Typhi, which does not contain a virulence plasmid. The essential virulence region is contained within a ca. 8-kb region containing *spv* (salmonella plasmid virulence) genes. Of these genes, *spvB* encodes a protein that ADP-ribosylates actin causing destabilization of the cytoskeleton in host cells. The plasmids are not essential for the production of enteritis, but some plasmids contribute to the initial intestinal attachment stage of systemic disease (Rychlik et al. 1998; Clayton et al. 2008). It may be that this region acts

as a switch, and the nature of any interaction with genes in SPI-2 and other pathogenicity islands would be interesting.

GENOME STRUCTURE WITH REFERENCE TO VIRULENCE

The current classification of *Salmonella* is extremely complex. Based on DNA–DNA hybridization results, the genus *Salmonella* is divided into major two species: *S. enterica* and *S. bongori* (fig. 14.2). *S. enterica* is then further divided into six distinct subspecies (I, II, IIIa, IIIb, IV, and VI), based on biochemical differences. The genus *Salmonella* is further divided by serology into over 2500 serovars using the Kauffman and White scheme. This classification scheme defines the serogroup according to expression of somatic LPS O antigens, and the serovar by expression of flagellar H antigens.

Features of the four *Salmonella* serovar genomes which were first sequenced are shown in table 14.1; the four serovars are very similar in size and gene numbers. This observation is extended if these whole genome sequences are aligned which shows that the genomes are remarkably conserved, displaying a high degree of overall synteny with all of the genomes being essentially colinear except for several large-scale rearrangements around the origin or terminus of replication. This is the most common form of chromosomal rearrangement separating related bacterial genomes. The reciprocal inversion event seen in *S.* Typhi is well documented and is the result of recombination between rRNA operons which are the most likely sites for recombination to occur (Tillier and Collins 2000).

Interestingly, if *Salmonella* and *E. coli* are aligned, a high degree of order is still maintained

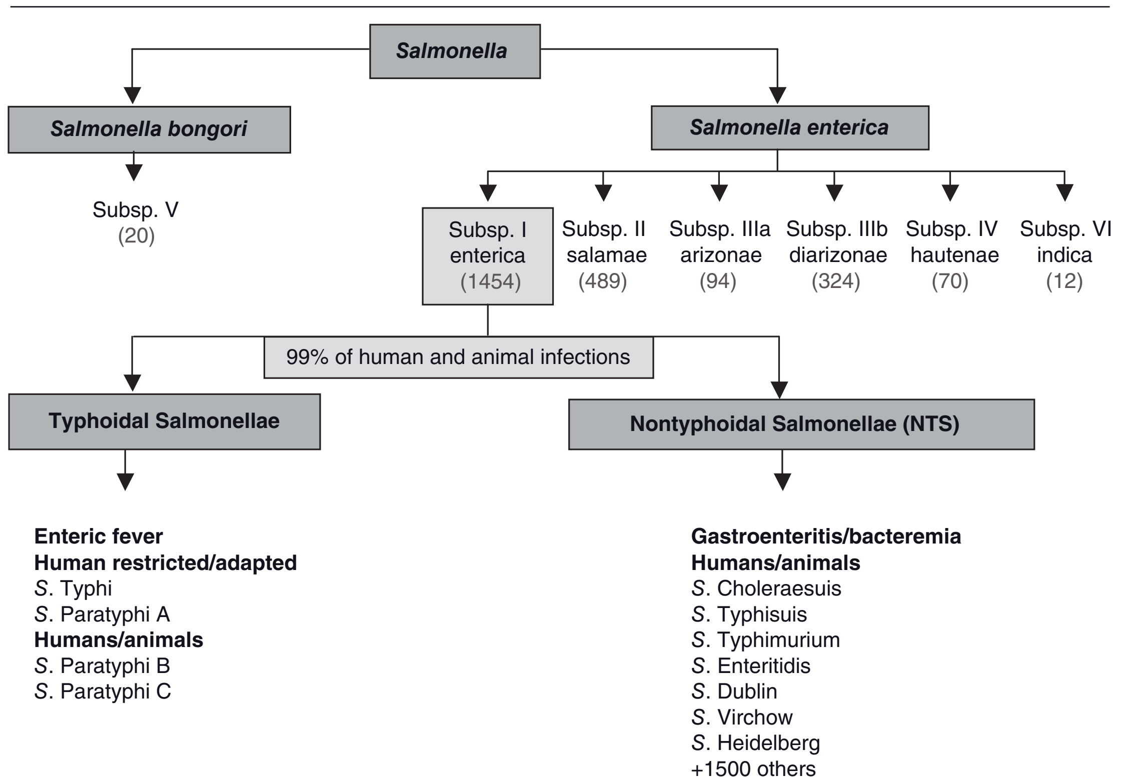

Figure 14.2. The genus *Salmonella*. *Salmonella* subspecies have been defined by multilocus enzyme electrophoresis (MLEE), DNA–DNA hybridization, 16S RNA analysis, and biotyping. Numbers in brackets indicate the total number of serotypes included in each subspecies. Common serotypes are listed, but other serotypes may cause bacteremia or focal infection. subsp.—subspecies. (Figure adapted with permission from Langridge et al. 2008).

Table 14.1. General Properties of *Salmonella enterica* Serovar Genomes

Serovar	Typhimurium	Typhi	Choleraesuis	Paratyphi A	Enteritidis	Gallinarum
Strain	LT2	CT18	SC-B67	SARB42	P125109	287/91
Size (bps)[a]	4,857,432	4,809,037	4,755,700	4,585,229	4,685,848	4,658,697
% G + C	52.22	52.09	52.11	53	52.17	52.20
N° CDSs	4451	4599	4445	4263	4318	4274
Coding density	86.8%	87.6%	83.9%	82.5%	85.5%	79.9%
Average gene size (bps)	947	958	898	924	953	939
rRNA operons	7	7	7	7	7	7
tRNA	85	78	85	82	84	75
Pseudogenes[b]	25	204	151	173	113	309
Plasmid(s)	1	2	2	0	2	2

[a] Base pairs.
[b] As detailed in the original publications.

even after a separation estimated at 100–140 million years ago (Ochman and Wilson 1987; Doolittle et al. 1996), consistent with the notion that the enteric genome architecture was fixed at an early time point in enteric evolution and prior to the adaptation to different hosts seen in *Salmonella*.

The dramatic expansion of insertion sequence (IS) element numbers observed in *Salmonella* and subsequent recombination between these elements is thought to be the result of the organism having been through a recent evolutionary bottleneck: conditions which are thought to increase the fixation of mutations by genetic drift and associated with a switch in lifestyle (Andersson and Hughes 1996).

Despite their taxonomic similarity, shared gene content for members of the same bacteria species, such as *E. coli,* can be as little as 40% when comparing pathogenic and nonpathogenic isolates (Welch et al. 2002). The core gene set shared by 11 *Salmonella* strains was composed of approximately 2500 orthologous genes, and if *E. coli* and *Shigella* strains were introduced into the comparison then the number was reduced to approximately 2000 orthologous genes (Vernikos et al. 2007).

It seems likely that the majority of the horizontally acquired genes in the *Salmonella* genomes were acquired early in their evolution. With regard to *S. enterica*, these genes are shared between most of the lineage since 60%–70% of the putative horizontally acquired (PHA) genes were inserted following the divergence of *Salmonella* from the *E. coli* lineage but prior to the divergence of the *S. enterica* subspecies. This includes many of the key SPIs (see below) such as SPI-1 to SPI-5 and that these events mark the key acquisition of genes that enabled the exploration of new niches. These include a large number of genes involved in general metabolism, such as the *cob* operon of *S. enterica*, which encodes vitamin B_{12} biosynthesis and has been previously shown to be horizontally acquired in the *Salmonella* lineage following its divergence from the *E. coli* lineage (Lawrence and Roth 1996). The *cob* genes are thought to have been lost from the enterics early in their evolution but reacquired by *Salmonella* ~71 million years ago following the split of the *S. arizonae* from the *S. enterica* lineages (Lawrence and Roth 1996).

The difference in the extent of phage related functions in *S.* Typhi and Paratyphi from Typhimurium, Enteritidis, and Gallinarum suggests that recent evolutionary time has been dominated by the acquisition of bacterial viruses (prophage; see below) but that earlier evolutionary events in *Salmonella* were dominated by the acquisition of metabolic and virulence functions.

In Silico Analysis of the Accessory Genome

In whole genome comparisons of *S.* Typhi and *S.* Typhimurium, this core genome comprises ~87% of its coding capacity (McClelland et al. 2001; Parkhill et al. 2001). The core genome is thought to encode essential metabolic and colonization functions that facilitate the general enteric lifestyle. The core regions are interdispersed with regions that are

either unique to that isolate or of restricted phylogenetic distribution, termed the accessory genome. It is thought that these accessory regions are enriched for functions that could explain differences in host range and pathogenic potential of the different salmonellae.

Much of the accessory genome is composed of clusters of three genes or more. The largest individual accessory gene clusters are represented by integrated prophage and SPIs which in *S.* Typhi range from ~10 to >130 kb. However, less visible are the large number of small gene insertions/deletions. When comparing *S.* Typhi with *S.* Typhimurium, there are 145 small insertions/deletions of 10 genes or less compared with 12 events of 20 genes or more. Interestingly, there are very few (42) single gene insertions/deletions which are unique to *S.* Typhi compared with *S.* Typhimurium. Many of the smaller insertions/deletions of less than 10 genes encode functions such as toxins or adhesins, but more commonly these regions encode chaperone-usher fimbrial operons, sugar transport/metabolism systems, and restriction-modification systems.

The genomes of *S.* Enteritidis and *S.* Gallinarum resemble each other more than they do *S.* Typhimurium in terms of shared genes.

Microarray Studies Quantifying Genome Variation

Using a microarray largely based on *S.* Typhimurium LT2, Porwollik et al. (2004) showed that 21% of *S.* Typhimurium genes were absent in at least 1 of 79 strains of *Salmonella* subspecies I tested. Surprisingly, these strains included recent clinical isolates. Among the regions most frequently missing from different serovars were the LPS-O antigen polymerase genes (likely to be divergent rather than missing), the genes responsible for the expression of the phase 2 flagella, *ratB,* involved in fecal shedding, and genes that encode sugar and multidrug transport proteins (Chan et al. 2003; Porwollik et al. 2004).

In many cases, such as with Typhimurium, Typhi, and Enteritidis, isolates from the same serovar show a conserved genomic signature and so are considered monophyletic. However, other *Salmonella* serovars show significant genome variation between isolates of the same serovar, and despite being coclassified by serology, the lineage is considered to be polyphyletic in nature (i.e., not all of the isolates of the same serovar show the same genomic signature). Serovars that appeared to be polyphyletic include Dublin, Saint Paul, Infantis, Muenster, and Paratyphi B (Porwollik et al. 2004). In addition, these analyses also confirmed previous notions that there are close genetic relationships between some serovars such as between *S.* Enteritidis and *S.* Dublin or *S.* Paratyphi C and *S.* Cholerasuis (Chan et al. 2003; Porwollik et al. 2004).

Genomic Islands, *S*PIs, and Islets

Horizontally acquired DNA sequences that contain functionally related genes with limited phylogenetic distribution, that is, present in some bacterial genomes while being absent from closely related ones, are often referred to as pathogenicity islands or genomic islands (GIs). These mobile elements are often inserted alongside tRNA genes and have direct repeats (DRs) and mobility genes (e.g., integrase, transposase), which has led to a definition of the GI structure that includes these features (Hacker et al. 1997; Hacker and Kaper 2000; Schmidt and Hensel 2004). Traditionally, GIs in *Salmonella* that carry genes associated with virulence are termed SPIs. There are now 17 published SPIs predicted or known to encode a range of functions including exopolysaccharide, TTSS and their associated effector proteins, type four secretion systems (T4SS), and metal uptake systems, in addition to many proteins of unknown function (Table 14.2). It is apparent that most SPIs were acquired early on in the evolution of *S. enterica,* being present in all of the sequenced isolates. However, there are exceptions to this; SPI-7, SPI-8, and SPI-15 appear to be more recent acquisitions, with, for example, SPI-7 being restricted to *S.* Typhi isolates and some isolates of *S.* Paratyphi C and *S.* Dublin (Pickard et al. 2003).

Since SPI-7 is a good example of the structure and features of SPIs, it can be used as an exemplar. SPI-7 encodes the *viaB* locus encoding the Vi capsular polysaccharide (Hornick et al. 1970; Robbins and Robbins 1984; Hashimoto et al. 1993). The G + C content of SPI-7 is 49.7% (slightly lower that the genome average G + C content of Typhi CT18 at 52%). The overall gene density of SPI-7 is 0.99 genes/kb, while the genome average is 0.91 genes/kb. SPI-7 is inserted at the 3′ end of the $tRNA^{Phe}$ gene which has been displaced at the 3′ end of SPI-7; the insertion has fully restored the displaced DNA fragment of the tRNA at the point of insertion. Like many SPIs such as SPI-2, SPI-6, and SPI-10, the sequence composition and gene content of SPI-7 is

Table 14.2. *Salmonella* Pathogenicity Islands (SPI)

Name	Description	Size and features	Original reference
SPI-1	TTSS, iron uptake	40 kb	Hansen-Wester and Hensel (2001)
SPI-2	TTSS	40 kb, tRNA-*valV*	Hensel (2000)
SPI-3	*mgtCB* Mg^{2+} transport	17 kb, tRNA-*selC*	Blanc-Potard et al. (1999)
SPI-4	Type I secretion and large repetitive protein	23 kb	Wong et al. (1998); Parkhill et al. (2001)
SPI-5	TTSS effectors	8 kb, tRNA-*serT*	Wood et al. (1998)
SPI-6	*saf tcs* fimbrial systems	59 kb, tRNA-*aspV*	Parkhill et al. (2001)
SPI-7	Vi antigen and the SopE prophage	134 kb, tRNA-*pheU*	Parkhill et al. (2001)
SPI-8	Bacteriocin immunity protein	6.8 kb, tRNA-*pheV*	Parkhill et al. (2001)
SPI-9	Type I secretion and large repetitive protein	16 kb	Parkhill et al. (2001)
SPI-10	Prophage ST46, *sef* fimbrial operon	33 kb, tRNA-*leuX*	Parkhill et al. (2001)
SPI-11	TTSS effectors and PhoPQ-activated proteins	14 kb	Chiu et al. (2005)
SPI-12	*msgA* and *narP*	6.3 kb	Chiu et al. (2005)
SPI-13	Required for survival in chicken macrophages	~7 kb, tRNA-*pheV*	Shah et al. (2005)
SPI-14	Unknown	~11 kb	Shah et al. (2005)
SPI-15	Proteins of unknown function	6.5 kb, tRNA-*gly*	Vernikos and Parkhill (2006)
SPI-16	Bacteriophage remnants and LPS modification genes	4.5 kb, tRNA-*arg*	Vernikos and Parkhill (2006)
SPI-17	Bacteriophage remnants and LPS modification genes	5.1 kb, tRNA-*arg*	Vernikos and Parkhill (2006)
SGI-1[a]	Multiple antibiotic resistance genes	43 kb	Boyd et al. (2001)
HPI[b]	Iron uptake in *Yersinia*	ND	Oelschlaeger et al. (2003)

[a] *S.* Typhimurium DT104 only.
[b] High-pathogenicity island.
ND—not determined.

highly mosaic with distinct functional modules that were likely to have been acquired in more than one event (Pickard et al. 2003).

Unusually, inserted within SPI-7 is a prophage $\Phi SopE_{st}$ (33.5 kb in size) encoding the SPI-1 effector protein, SopE, which is important for *Salmonella* invasion (Wood et al. 1996; Mirold et al. 1999; Friebel et al. 2001). This prophage element probably represents an independent horizontal gene transfer event in Typhi, given that it is absent from SPI-7 in Dublin and Paratyphi C (Pickard et al. 2003).

Many SPIs lack identifiable repeat elements flanking their boundaries, and mobility genes, for example, integrases, suggesting that they may have been mislabeled as GIs and simply represent genomic regions deleted in other lineages. These SPIs are probably mobile elements that have stably integrated in the *Salmonella* chromosome and may be ancient insertions which have, over time, lost their ability to mobilize and the features associated with that.

Other regions in the genome are known as genomic islets showing a variable distribution and generally displaying none of the features associated with GIs bar anomalous nucleotide composition. Examples of these regions in *Salmonella* include genes associated with LPS and capsular polysaccharide (Stevenson et al. 1991; Reeves 1993). In the case of LPS, over 60 forms of O antigen are known in *S. enterica.*

All of the published *Salmonella* genomes are polylysogenic carrying between three to five integrated bacteriophages (prophages) as well as several prophage remnants. In addition to contributing to

the overall DNA diversity differentiating both *Salmonella* lineages and isolates, bacteriophage can also act as vectors carrying "cargo genes" that are not essential for phage proliferation, but which can impinge directly on the pathogenic potential of their host. The phenomenon of lysogenic conversion is well described within *S. enterica* serovars including *S.* Choleraesuis (Barrow 1986) and *S.* Typhimurium, which harbours several lysogenic bacteriophage: Gifsy-1, Gifsy-2, Fels-2, and SopE (Stanley et al. 2000; Figueroa-Bossi et al. 2001). The genetic cargo of these prophages include the *sopE* gene, encoding a TTSS effector protein shown to stimulate GDP/GTP nucleotide exchange in several Rho GTPases *in vitro* (Hardt et al. 1998b), and *sodCI*, a Cu/Zn periplasmic SOD that protects the host against oxidative stress (Figueroa-Bossi and Bossi 1999).

Many of the *S.* Typhimurium prophage cargo genes have been shown to increase the pathogenicity of *S.* Typhimurium in various models including mice, cattle, and macrophage (Farrant et al. 1997; Figueroa-Bossi and Bossi 1999; Figueroa-Bossi et al. 2001). For example, curing *S.* Typhimurium strains of Gifsy-2 results in a >100-fold attenuation of virulence in a mouse model of disease (Figueroa-Bossi and Bossi 1999). Genome analysis has also shown evidence that prophages have had a long-term role in *Salmonella* evolution. Several *Salmonella* virulence determinants such as the TTSS effector protein SspH2 and the PhoPQ-activated genes *pagKMO* (Gunn et al. 1998; Miao et al. 1999) and various LPS modification genes are surrounded by prophage gene remnants.

Functional Gene Loss: Pseudogenes

Along with the acquisition of genes, prophage, and GIs, it is clear that gene loss has had and continues to have an important role to play in the fluidity of the *Salmonella* genome. Pseudogenes are defined as being untranslatable due to the presence of stop codons, frameshifts, internal deletions or through disruption following the insertion, for example, of an IS element. There are always dangers to *in silico* prediction of pseudogenes and from assuming that a gene function has been lost when a significant part of the coding sequence has been deleted.

Analysis of the *S.* Typhi genome revealed over 200 pseudogenes, while *S.* Typhimurium was predicted to contain only around 39 (McClelland et al. 2001; Parkhill et al. 2001). Moreover, it is clear that the pseudogenes in *S.* Typhi are not randomly spread throughout the genome: they are overrepresented in genes that are *Salmonella* specific when *S.* Typhi is compared with *E. coli* (59% of the pseudogenes lie in the unique regions, compared with 33% of all *S.* Typhi genes being unique), and many of the pseudogenes in *S.* Typhi have intact counterparts in *S.* Typhimurium that are involved in virulence and host interaction (Parkhill et al. 2001).

Pathogenicity determinants lost by both *S.* Typhi and *S.* Paratyphi A include genes encoding the TTSS effector proteins: SopA, SlrP, SopD2, and SseJ. Additionally, *S.* Paratyphi A carries mutations in *sifB* and *sspH2,* while *S.* Typhi has lost a functional copy of *sopE2*. These effectors are associated with causing diarrhea or systemic disease in other *Salmonella* serovars. Consequently, their loss is consistent with *S.* Typhi and *S.* Paratyphi A rarely causing diarrhea. The loss of these virulence-associated proteins may actually prolong and moderate the infection, thereby increasing transmission. Other important functions lost by *S.* Typhi and *S.* Paratyphi A include components of multiple chaperone-usher fimbrial systems and *tar* encoding a chemotaxis receptor protein, which if deleted in *S.* Typhimurium leads to a hyperinvasive phenotype.

Like *S.* Paratyphi A and *S.* Typhi, *S.* Choleraesuis rarely causes diarrhea, and in common with them it possesses pseudogenes involved in shedding and colonization of the gut as well as potential pathoadaptive mutations in chemotaxis signal transduction pathways that increase invasiveness of the bacterium (Chiu et al. 2005). The large number of pseudogenes (309) in *S.* Gallinarum bear a similar relationship to *S.* Enteritidis (113) as Typhi does to Typhimurium and include the same types of genes affecting metabolism (glucarate, hydrogenase, propanediol, and cobalamin, glycogen, and amino acid catabolism and biosynthesis) (Thomson et al. 2008).

In contrast to other organisms containing multiple pseudogenes such as *M. leprae* (Cole et al. 2001), most of the pseudogenes in these salmonellae are caused by a single mutation, suggesting that they have been inactivated relatively recently. This is consistent with the fact that *S.* Typhi is clonal and thought to have arisen as a serovar only within the last 50,000 years (Kidgell et al. 2002).

There are patterns of gene loss with the loss of function in different genes encoding the same trait. For example, both *S.* Typhi and *S.* Paratyphi A possess mutations in cobalamin (vitamin B_{12}) biosynthesis, the propanediol utilization pathway,

hydrogenase 1, and the fimbrial clusters *bcf* and *saf* (Parkhill et al. 2001; McClelland et al. 2004). In *S.* Typhimurium, 1,2-propanediol is an important source of energy and B_{12} is an essential cofactor for 1,2-propanediol degradation. Furthermore, *S.* Typhimurium mutants unable to use 1,2-propanediol are significantly attenuated in their ability to grow in macrophages (Klumpp and Fuchs 2007).

The ability of serovars such as Typhimurium (but not Gallinarum or Typhi) to use molecular hydrogen as an energy source is associated with the production of membrane-bound hydrogenases, including hydrogenase 1 (Hyd1). Although the exact physiological role of Hyd1 is equivocal, *S.* Typhimurium Hyd1 mutants are affected in virulence (Maier et al. 2004). The presence of these mutations within Gallinarum and Typhi suggests that they may be characteristic of more invasive *Salmonella* serotypes. Consistent with this, some of these common traits have also been noted to have been lost in representatives of systemic (*Yersinia pestis)* versus gut adapted (*Yersinia enterocolitica*) versus yersiniae, and again, in this system gene loss may be involved in the adaptation from a gut to a systemic lifestyle (Thomson et al. 2006).

IMMUNITY TO INFECTION AND ITS MANIPULATION BY *SALMONELLA*

The following is a brief overview of immunity to *Salmonella* infections. The use of the mouse as a model host for studying immunity to *Salmonella* and as a paradigm of an intracellular pathogen has provided valuable insights into the cellular and humoral interactions that form the basis of innate and adaptive protection. The unique aspects to infection in other host species suggest that the mouse may form a generic model against which particular aspects of immunity in other species can be compared. More comprehensive reviews of immunity to *Salmonella* and other species can be found in Davison et al. (1996), Wigley et al. (2004), Mastroeni (2005), and Chappell et al. (2008).

The response to the pathogen is characterized by an initial innate response which involves interaction between bacterial components and epithelial cells and M cells, usually in the gut. In the usual model studied, namely *S.* Typhimurium infection in mice or chickens, these interactions result in the production of strong proinflammatory chemokines, particularly IL-1, IL-6, and IL-8 or its equivalents. These lead to infiltration into the site of invasion by neutrophils/heterophils and macrophages which initiate the process of a strong adaptive response. Both epithelial cells (Paneth cells) and neutrophils also generate high local concentrations of antimicrobial peptides (defensins), but whether *Salmonella* strains colonizing the gut manipulate their expression to avoid inhibitory effects is unclear.

The initial induction of proinflammatory cytokines is induced by the interaction with TLRs, in particular TLR-4, which interacts with LPS, and TLR-5, which interacts with bacterial flagellin. TLR-4 is easily triggered by LPS, but TLR-5 is expressed at the basolateral surface of epithelia cells and will only be activated as a result of bacterial invasion. Proinflammatory cytokine and chemokine production leads to neutrophil (heterophil in chickens) infiltration, which is a characteristic feature of *S.* Typhimurium infection in chicken, cattle, pig, and sheep, in addition to mice. The importance of these cells is also shown by the fact that their elimination in young chickens treated with 5-fluorouracil results in the transformation of *S.* Enteritidis infection from one largely limited to the intestine to a disease more closely resembling typhoid. In contrast, *in vitro* or *in vivo* infection with *S.* Gallinarum or *S.* Pullorum causes little activation, in some cases even downregulation, of proinflammatory cytokines and chemokines (Kaiser et al. 2000), and corresponds with the low-level heterophil infiltration into the gut following infection with these serovars. In addition, nonflagellate mutants of *S.* Typhimurium induce much lower levels of IL-1β, and SPI-1 mutants induce lower levels of IL-6. These differences are interesting and suggest that the development of systemic disease by typhoid serotypes involves the inhibition of a heterophil influx, which normally prevents the invasion of large numbers of *Salmonella*. The fact that both *S.* Gallinarum and *S.* Pullorum are the only nonflagellate serovars of *S. enterica* suggests that this may be a mechanism whereby these serovars are able to invade without generating a strong inflammatory response (Iqbal et al. 2005). In addition, other genes, such as *sipA*, are truncated in a number of serotypes associated with systemic infection, and this may also be related.

Little is known about the interaction between the different *Salmonella* which produce different forms of the disease (pathovars) and DC in food animals. In cattle exposed to live *S.* Typhimurium, bovine DC up-regulate expression of MHC-I, MHC-II,

CD40, CD80, and CD86 molecules on their cell surface. In contrast, besides a marginal up-regulation of CD40, macrophages do not exhibit detectable changes in their expression of cell surface molecules. Both macrophages and DC exposed to *S.* Typhimrium up-regulate mRNA transcription of TNF-α, IL-1β, IL-6, and iNOS. Up-regulation of mRNA transcripts for granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-12p40 occurs only in DC, while only macrophages up-regulate IL-10. As expected, DC induced stronger T cell responses than macrophages (Norimatsu et al. 2003).

In mice, the functions and recruitment of phagocytic cells at the foci of infection require the concerted action of several cytokines including TNF-α, IFNγ, IL-12, IL-15, and IL-18. TNF-α is involved in cell recruitment as well as regulation of reactive oxygen species (ROS)-mediated killing. In these early stages of the infection, IFNγ is produced presumably by natural killer cells in response to IL-12 and IL-18 and mediates the up-regulation of iNOS-dependent macrophage antibacterial mechanisms. Thus, the early induction of an inflammatory immune response is critical to the resolution of *Salmonella* infection (Mastroeni 2005). The generation of nitric oxide and ROS by avian macrophages in response to *Salmonella* infection has also been demonstrated.

In mice, resistance to salmonellosis is linked to the presence of a functional *Nramp1* gene in macrophages. In chickens, *Nramp1* plays a significant albeit minor role in resistance to systemic salmonellosis (Hu et al. 1997) in comparison with the autosomal dominant *SAL1* gene which is a major determinant of susceptibility to systemic disease, expressed through increased levels of oxidative burst in macrophages in resistant lines (Wigley et al. 2002). An unrelated gene(s) contributes to differences in susceptibility in intestinal infection in which resistance is also dominant, autosomal, and not related to MHC or *Nramp1* (Barrow et al. 2003). The role of *Nramp 1* in cattle is somewhat confusing. Resistance to *S.* Dublin and *S.* Typhimurium in cattle is not linked as they are in mice (Qureshi et al. 1996), which may reflect the similar disease produced in mice by these serotypes, whereas in cattle these serovars induce systemic and enteric diseases respectively.

The contribution of cellular and humoral immunity toward control of established *Salmonella* infections in mice has been discussed in detail elsewhere (Mastroeni 2005). The relative roles of cellular and humoral immunity in other animals has not been established. Antibody responses are detectable in chickens, pigs, and cattle and are frequently used for herd and flock monitoring. The role of antibodies in gut clearance is unclear. It might be thought that they would be essential through the effects of specific secretory mucosal IgA. However, surgical bursectomy of embryonic chickens results in birds hatching which are unable to produce antibodies. Such birds clear *S.* Typhimurium infections from their intestines at the same rate as birds which can produce antibody (Beal et al. 2006). Either gut clearance is a cellular function or specific antibody is required for T cell activity and mediating the antibacterial effects of macrophages. Either way, this result is fascinating. Chemical bursectomy, which affects several different cell types including T cell maturation, results in inability to clear infection from the gut (Desmidt et al. 1998).

Infections with virulent and attenuated strains of *S.* Enteritidis in young chicks lead to an influx of T lymphocytes into the ileum and the ceca around 20–24 h post infection, with a later influx of B lymphocytes (Van Immerseel et al. 2002). Reproductive tract infection by *S.* Enteritidis leads to a surge in T lymphocytes in the ovaries and oviduct, reaching a peak at 10 days post infection, while a peak in B cells occurs at 14 days post infection. Increased percentages of $CD4^+$ and $TCR1^+$ $CD8^+$ T cells were observed in peripheral blood during infection of 1-day-old chicks with *S.* Typhimurium. T lymphocyte proliferation can be detected in cells isolated from the spleen following infection with *S.* Typhimurium or following vaccination with killed or live vaccines. The highest levels of proliferation are found around 3 weeks post infection, a time that correlates with clearance from the gut and of the transient systemic infection found with the invasive *S.* Typhimurium strain used in that study.

Inoculation of cattle with live attenuated *S.* Dublin or *S.* Typhimurium induces the development of antigen-specific cell-mediated immune responses that can be detected in peripheral blood (Villarreal-Ramos et al. 1998). These responses are assumed to be mainly from $CD4^+$ T cells due to the exogenous nature of the antigens used for *in vitro* stimulation. The role of $CD8^+$ T cells in immunity to *Salmonella* is largely unstudied in domestic species. In mice, protective immune responses elicited by vaccination are dependent on the presence of $CD4^+$ and $CD8^+$ T

cells, and depletion of any of these cell subsets prior to challenge results in an increased susceptibility to challenge with virulent *S*. Typhimurium. Protection of cattle against challenge seems to be dependent on $CD4^+$ T cells, which presumably are able to provide help to B cells to synthesize antibodies.

The timing of IFNγ production together with antigen-specific T cell proliferation and the appearance of specific antibody in chickens which are beginning to clear systemic infection suggests that this involves primarily a Th1-type response. The basis for the absence of immune clearance in animals which show persistent infection is not known. This is seen with *S*. Typhi in man and *S*. Pullorum in poultry, although *S*. Dublin is also thought to enter the carrier state. Recent studies (Chappell et al. 2008) suggest that in chickens, *S*. Pullorum induces a Th2-type response as opposed to the more typical Th1-type response induced by serovars such as *S*. Typhimurium and *S*. Enteritidis, characterized by strong early expression of IL-6, CXCLi1, and CXCLi2 and production of IFNγ. IFNγ and IL-18 production is suppressed by *S*. Pullorum, and IL-4 levels are increased in a similar way to lepromatous cases of *M. leprae* infection. The bacterial characteristics that are associated with this phenomenon are unknown. Mutants of *S*. Pullorum with defective SPI-2 function do not enter the carrier state, but they are in fact unlikely to persist within macrophages long enough to be able to. Nonetheless, it seems likely that persistent intracellular infection is related directly or indirectly to SPI-2 function.

The persistence of small numbers of bacteria in the spleen of carrier birds changes at the onset of sexual maturity associated with the increase in concentrations of circulating sex hormones. This results in suppression of general and antigen-specific T cell responsiveness accompanied by bacterial multiplication in the spleen with spread to other organs, especially the reproductive organs and ovaries from whence they are transmitted to the next generation via the egg (Wigley et al. 2005). This occurs in females but not in males. *S*. Dublin produces a similar effect through infection of the fetus, which can result in fetal death and abortion. Whether in all carrier animals the overall T cell response is affected or whether the intracellular bacteria are invisible to T cells as a result of downregulation of MHC expression, as occurs in mice, is unclear. There is, however, potential here for immune manipulation to increase clearance.

CONCLUSION—PROBLEMS AND OPPORTUNITIES

The bacterial and host basis of the infection biology of this genus has been studied intensively for several decades as a result of the economic and public health importance of *Salmonella* infections. Despite the accumulation of vast amounts of information on the role of bacterial genes/proteins in the various stages of the infection and disease process, compounded more recently by the exponentially expanding information on genomic composition which will, itself, generate further information, much remains to be determined about virulence, immunity, and mechanisms to control these infections.

It is clear from the genome sequences that are available that not only are there great differences between serovars which express different forms of disease and pathology but that significant differences also exist between strains of the same serovar. Further sequencing of strains within serovars and pathological types (pathovars) will assist in understanding the population structure of these types and of the genus as a whole. This linked to further mass mutations systems such as transposon-mediated differential hybridization (Chaudhuri et al. 2009) and microarray analysis will also assist in identifying potential virulence genes and surface antigens which may be used as vaccine candidates or as serological markers which may be deleted in a live vaccine.

Many questions remain relating to virulence genes. The complex role of fimbriae in colonization and the attachment process remains to be determined. The absence of any clear association between individual fimbriae and virulence is puzzling but suggests that complex interactions perhaps relating to hierarchical patterns of expression may occur.

Much has been determined on the role of pathogenicity islands in systemic disease in the mouse model. How far this translates into systemic disease in other hosts species involving other serovars which differ so markedly in their accessory gene compliment remains to be seen, and it is clear that, although parallels exist, many differences can be found and the mouse model should be seen as a model against which other studies of infection biology can be compared. The role of individual genes within the different pathogenicity islands is slowly and painstakingly being determined. Although the genetic interactions of the *spv* genes

on the virulence plasmids have been determined, their exact role is unclear and the nature of the interaction with genes on pathogenicity islands which are important for systemic disease is intriguing.

Similarly, the murine model of *S.* Typhimurium infection has contributed enormously to understanding systemic salmonellosis and immunity in general, but many aspects peculiar to infections in cattle and poultry must be determined in these species and, in some cases, can be more useful (Beal et al. 2006). The nature of the carrier state can possibly be most clearly determined using poultry as a model, and approaches to clearance of persistent infections would be a great advance in disease control.

Although one could argue that in many cases of human and animal salmonellosis, a combination of hygiene and herd/flock management advocated by many government departments and international organizations, can go a long way toward preventing *Salmonella* infections, they are unable to prevent all aspects of the disease. New information undoubtedly generates the potential for new approaches to limiting infection and disease, and both approaches must be adopted to ensure that this perennial and ubiquitous pathogen remains under control.

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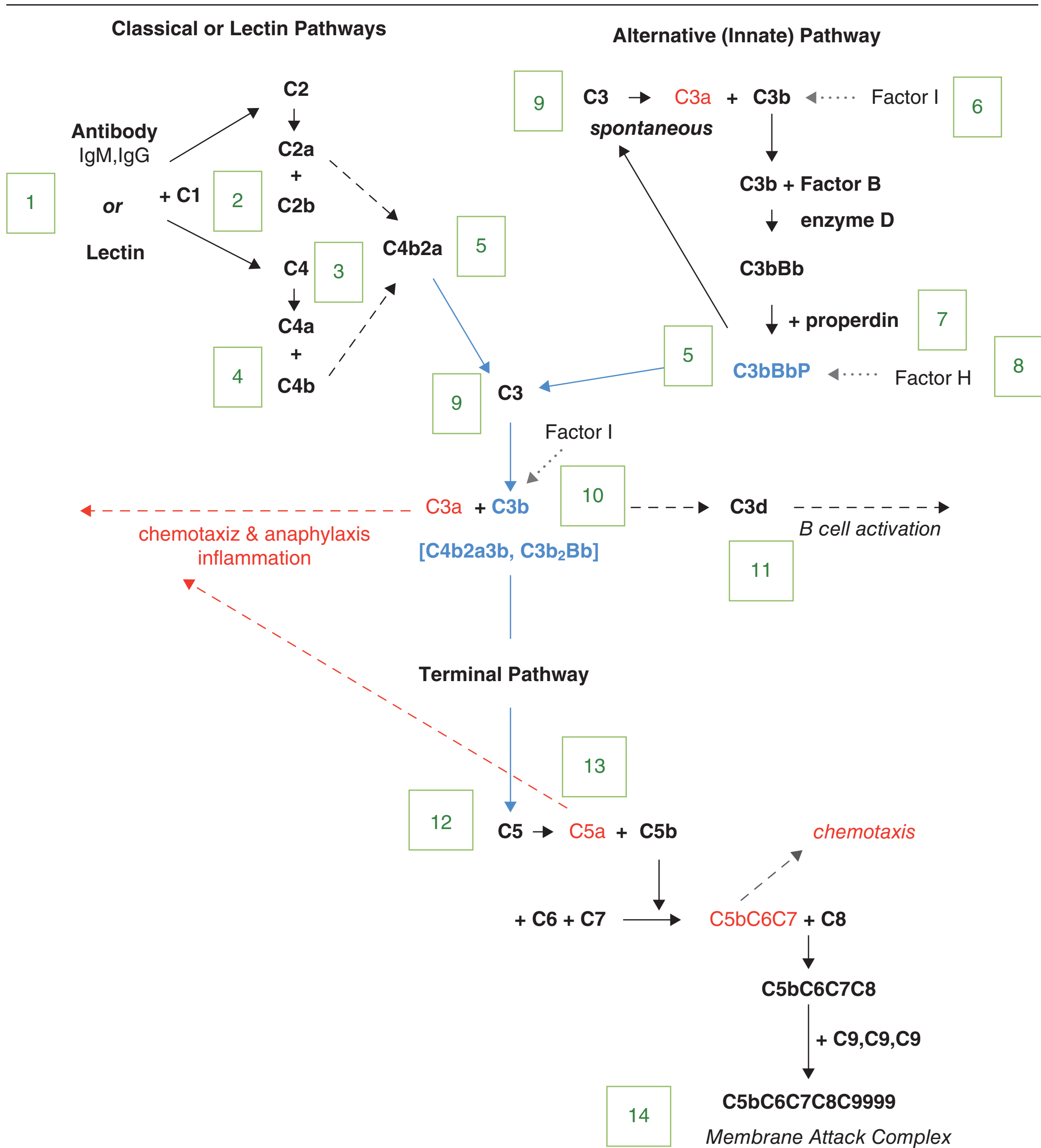

Figure 2.1. The complement cascade and microbial interference with complement activity. Numbers in boxes correspond to points vulnerable to microbial interference as indicated in table 2.2. C3 and C5 convertases are shown in blue. Factors in red are soluble split products associated with inflammation. Regulatory factors are shown in gray.

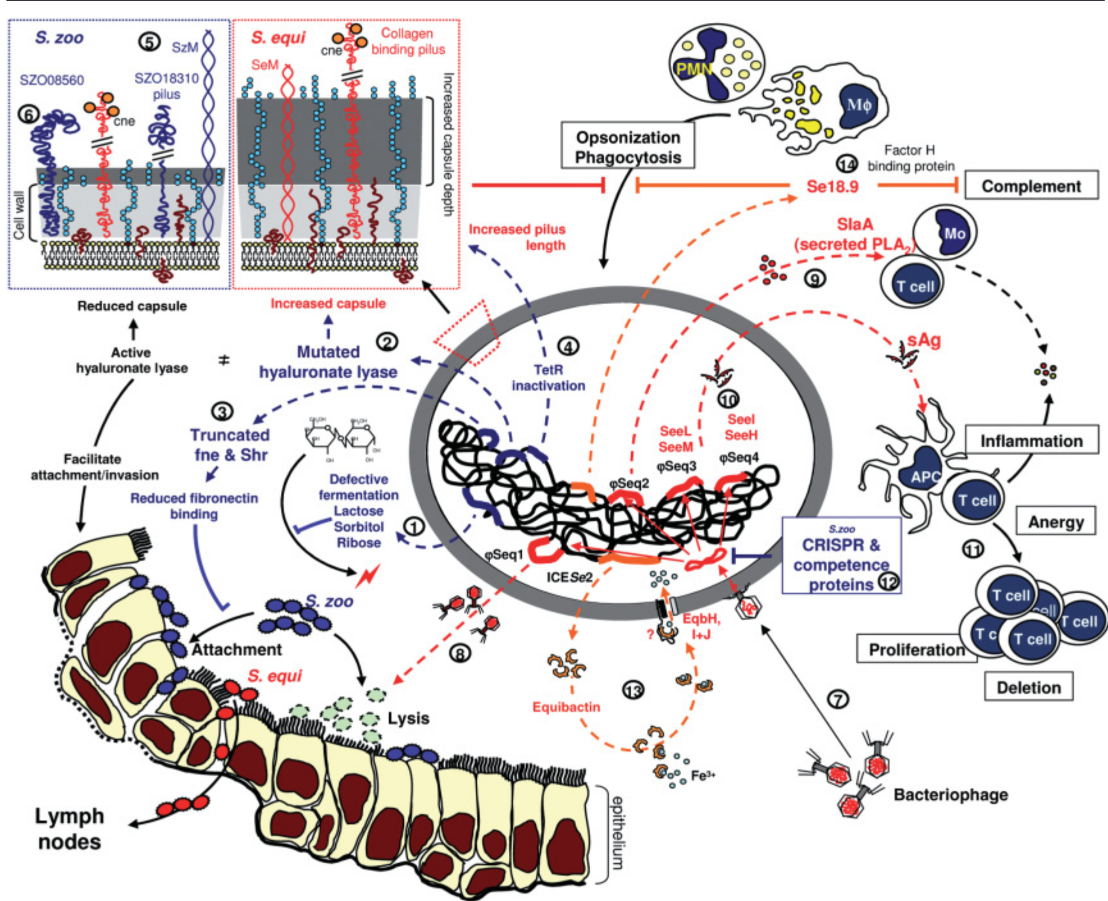

Figure 4.1. Hypothetical illustration showing genes and functions gained (red) and lost (blue) in the course of evolution of *S. equi* from the putative ancestral *S. zooepidemicus*. The figure is based on a comparison of the genome sequences of *S. equi* 4047 and *S. zooepidemicus* H70, and the legend is modified from that in figure 2 of Holden et al. (2009). (1) Loss of the ability of *S. equi* to ferment lactose, sorbitol, and ribose with possible adverse effect on its mucosal colonization ability. (2) Reduced hyaluronate lysase activity and abundant capsule synthesis by *S. equi* increases resistance to phagocytosis. (3) Truncation of FNE and SBR proteins in *S. equi* may decrease fibronectin-dependent attachment. (4) A nonsense mutation in the *tetR*-like regulator of *S. equi* may be associated with enhanced pilus production and adhesion to cell receptors in equids. (5) Loss of the SZO18310 pilus locus in *S. zooepidemicus* from *S. equi* may reduce its colonization potential in multiple hosts. (6) Loss of expression by *S. equi* of homologue of SZO08560 protein, which carries four Pfam repeat domains involved in host cell invasion by bacteria, such as *Listeria monocytogenes.* (7)–(12) Four prophage acquired by *S. equi* 4047 introduced genes for pyrogenic exotoxins SeeH, I, L, and M and phospholipase A2 (SlaA) that enhance its virulence and modulate inflammatory and immune responses in horses. Resistance of most *S. zooepidemicus* to phage integration is due to the presence of short palindromic nucleotide repeats (CRISPR) and associated genes. These sequences have been deleted from *S. equi* as a sequel to recombination between insertion (IS) elements. (13) Acquisition of the potential siderophore equibactin, encoded by the integrative conjugative element ICESe2, may be responsible for the high rate of proliferation of *S. equi* in tonsil and lymph nodes under conditions of low iron availability. (14) Binding of complement control factor H by Se18.9 reduces phagocytosis of *S. equi*.

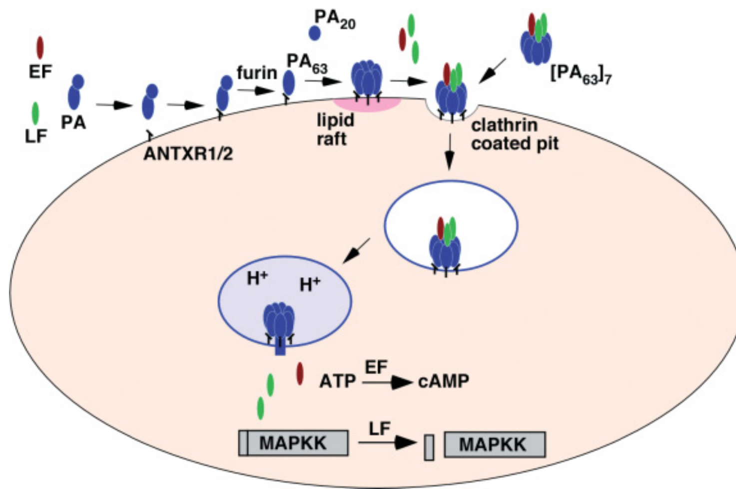

Figure 6.1. Entry of anthrax toxin into mammalian cells. The three components of anthrax toxin are released from the bacterium as soluble monomeric proteins: edema factor (EF), lethal factor (LF), and protective antigen (PA). PA binds the cellular receptors ANTXR1 and ANTXR2 and is then cleaved by furin-like proteases into PA_{20} and PA_{63}. PA_{63} forms heptamers, $[PA_{63}]_7$, that enter lipid rafts and bind up to three molecules of EF and LF. Alternatively, toxin complexes may form before they associate with cells. The complexes are internalized through clathrin-coated pits and are trafficked to acidic compartments (early endosomes and multivesicular bodies) that facilitate membrane insertion of $[PA_{63}]_7$ and translocation of EF and LF through the $[PA_{63}]_7$ pore. EF binds calmodulin and converts ATP to cAMP; LF is a protease that cleaves amino terminal fragments from MAPKKs. A co-receptor, LRP6, may participate in the entry process (not shown).

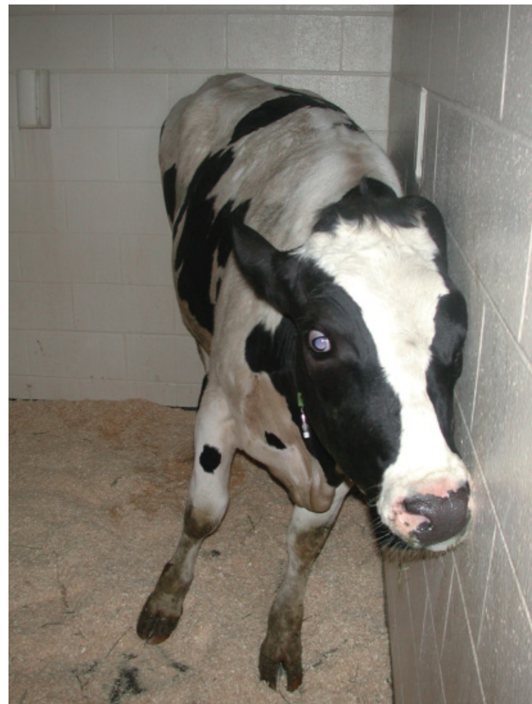

Figure 10.3. A 14-month-old Holstein heifer with *Listeria* meningoencephalitis ("circling disease"). Note the characteristic head tilt, ear droop, and leaning against the wall. The heifer circled to the right when induced to walk, and leaned on walls when standing. This animal was treated and survived.

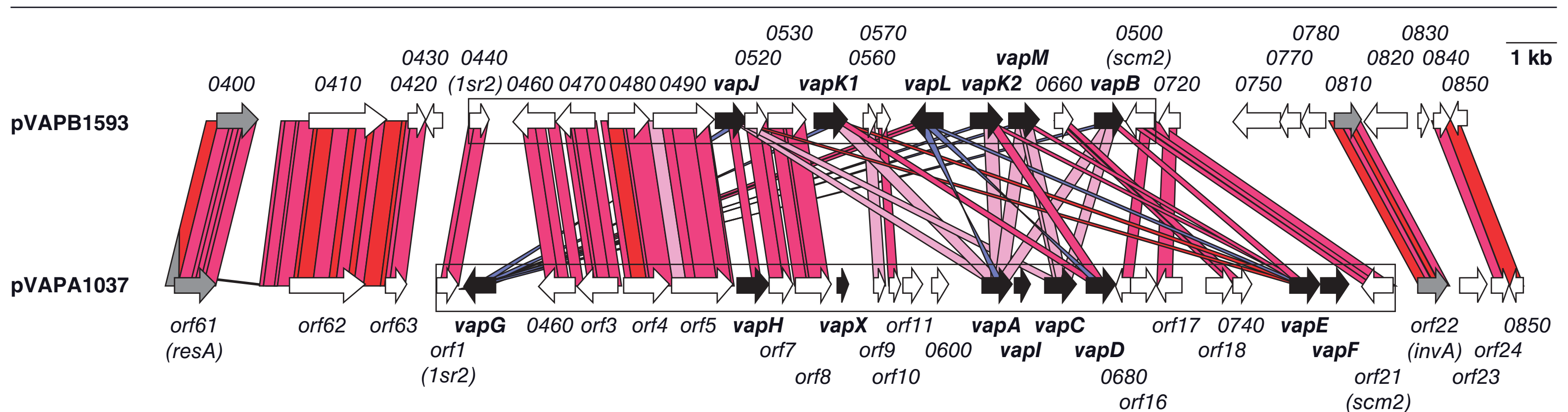

Figure 9.1. Genetic structures of the variable region of the *R. equi* virulence plasmids pVAPB1593 (pig type) and pVAPA1037 (horse type) and sequence comparision using the Artemis comparison tool (www.sanger.ac.uk/Software/Artemis/). Regions with significant similarity (tBlastx) are connected by colored lines (red, sequences in direct orientation; blue, sequences in reverse orientation). The intensity of the color indicates the strength of the sequence homology (pink/light blue, lowest; red/deep blue, highest). The virulence-associated *vap* genes are shown in black, and the mobility-related *resA*- and *invA*-like genes are shown in gray. The DNA region corresponding to the *vap* PAIs (boxed) were defined using the "Alien Hunter" algorithm (www.sanger.ac.uk/Software/analysis/alien_hunter/), which identifies horizontally acquired DNA by reliably capturing local compositional biases. Gene designations for pVAPB1593 according to standardized annotation nomenclature adopted for *R. equi* virulence plasmids (pVAP) (Letek et al. 2008b), those for pVAPA1037 according to the nomenclature used by Takai et al. 2000 (except for newly identified genes during the reannotation by Letek et al. 2008a). From Letek et al. (2008b), with permission, American Society for Microbiology.

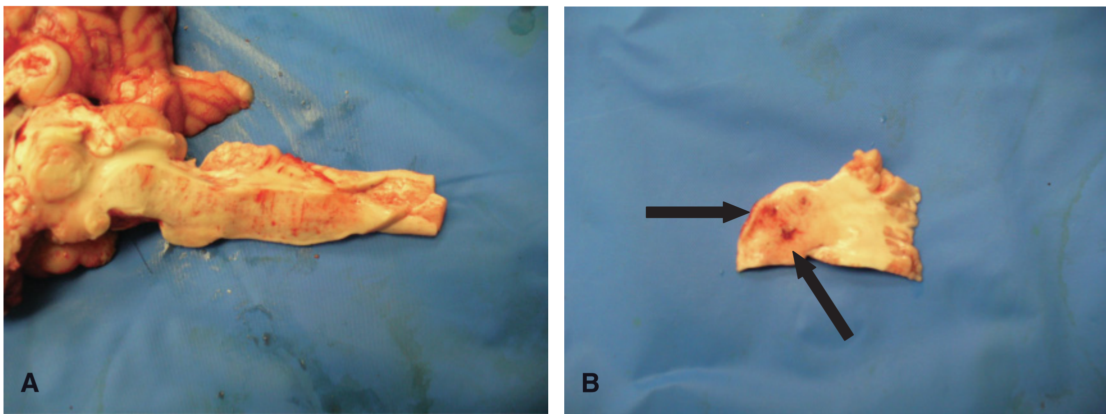

Figure 10.4. Sagitial section (A) and transverse section (B) of the brainstem of a 3-year-old Holstein cow that presented for neurologic dysfunction. Note the micro-abscessation associated with hemorrhages in the tissue (arrows).

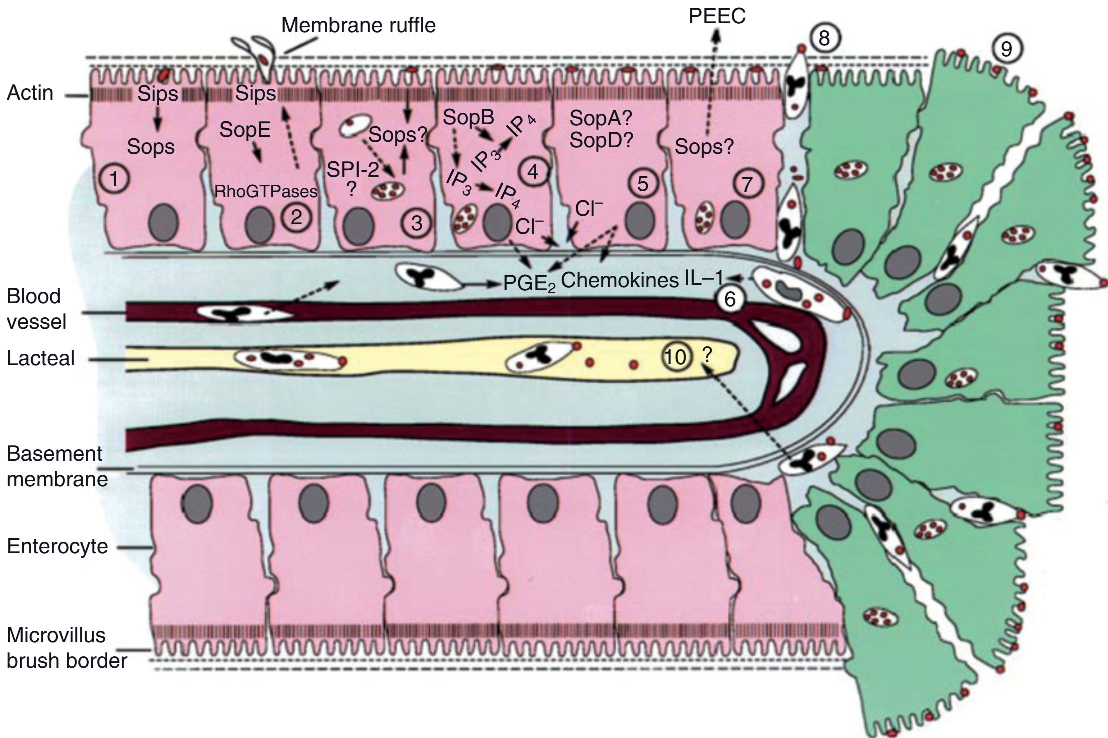

Figure 14.1. Schematic representation of stages of pathogenesis of enteritis. (1) Interaction with enterocytes, delivery of Sop proteins into cytoplasm via TTSS-1 and Sip-dependent pathway; (2) Sip proteins, SopE, and other Sops induce membrane ruffling inducing invasion; (3) bacteria in intracellular membrane-bound vesicles; (4) SopB affects inositol phosphate-related signaling events leading to electrolyte transport and fluid secretion; (5) infected epithelial cells secrete chemokines attracting inflammatory cells to foci of infection; (6) *Salmonella* interaction with inflammatory cells stimulating release of proinflammatory cytokines enhancing inflammation; (7) stimulation of trans-epithelial migration of granulocytes by *Salmonella*; (8) phagocytosis of *Salmonella* bacteria by inflammatory cells; (9) extrusion of infected enterocytes from villus surface leading to villus blunting and reduced fluid absorption; (10) migration of infected cells and bacteria to draining lymphatics carrying bacteria to systemic sites. (Figure used with permission)

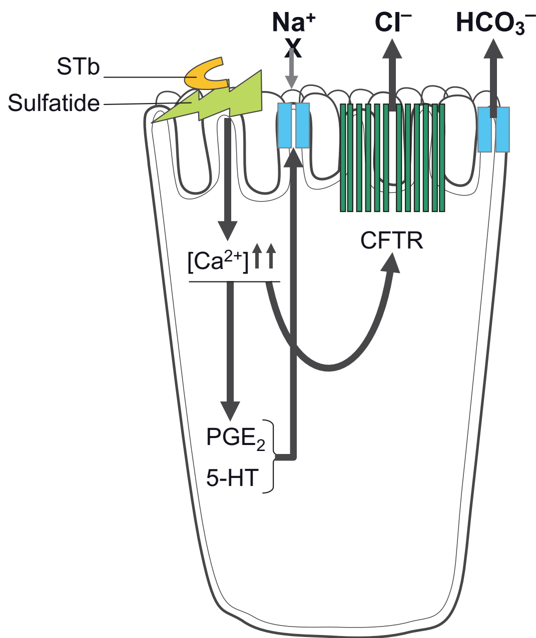

Figure 15.2. Mechanism of action of STb. Binding of STb to its receptor sulfatide leads to elevated intracellular levels of calcium. The high calcium levels lead to activation of the cystic fibrosis transmembrane conductance regulator (CFTR) and the secretagogues prostaglandin E_2 (PGE2) and 5-hydroxytryptamine (5-HT). These lead to secretion of Cl^- and HCO_3^- and inhibition of absorption of Na^+. (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal)

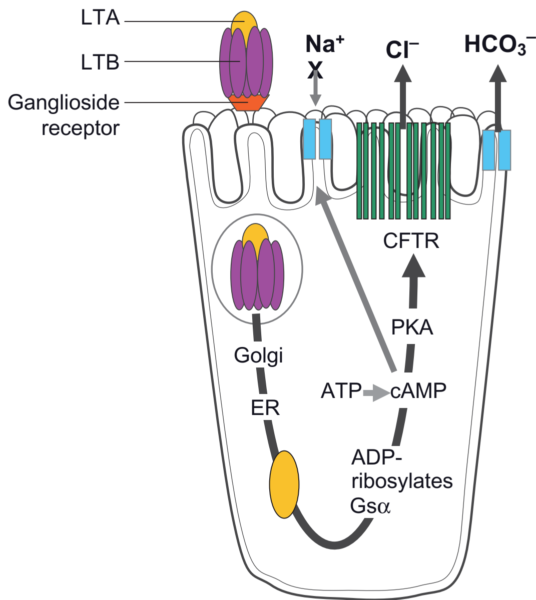

Figure 15.3. Major mechanism of action of LT. LT binds GM1 and other gangliosides, resulting in internalization by receptor-mediated endocytosis. Following transport to the Golgi and the ER, LTA1 is translocated to the cytosol where it ADP-ribosylates the regulatory protein Gsα which irreversibly stimulates production of cyclic AMP. Elevated levels of cAMP increase production of c-AMP-dependent protein kinase A, leading to phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR activation leads to secretion of Cl^- from secretory epithelial cells in the crypts, which stimulates HCO_3^- secretion from the Cl^-/HCO_3^- exchanger in villus epithelial cells (Sears and Kaper 1996; Viswanathan et al. 2009). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal)

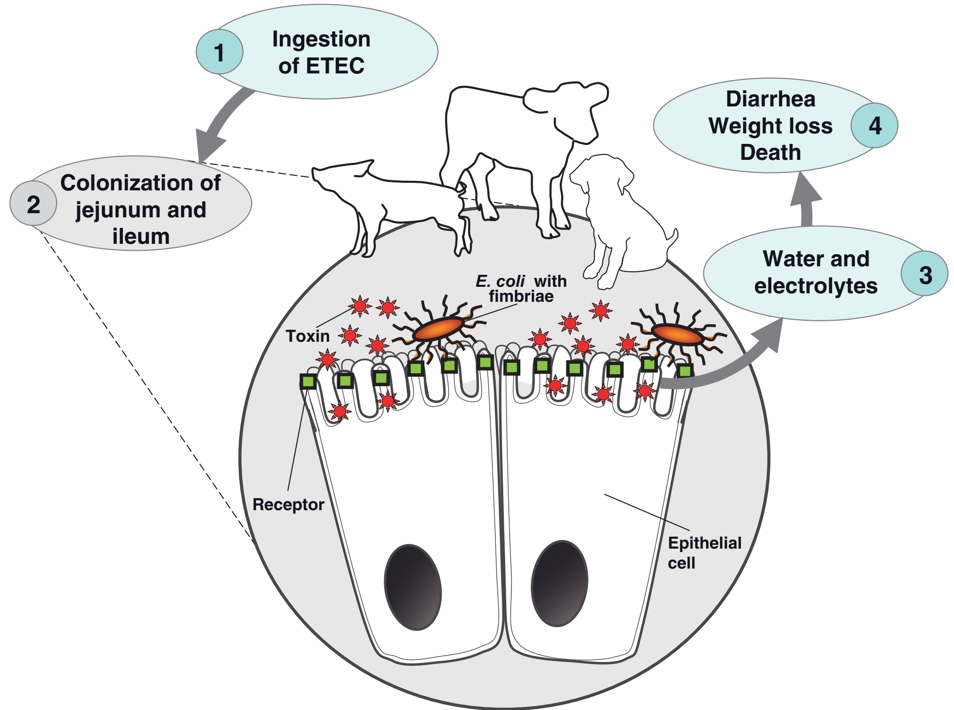

Figure 15.4. Schematic representation of the steps involved in the pathogenesis of ETEC infection. ETEC in the animal's environment are ingested (1), pass through the stomach, adhering to the small intestinal epithelium where they produce enterotoxins (2) that stimulate the secretion of water and electrolytes into the intestinal lumen (3). Loss of water and electrolytes leads to diarrhea, weight loss, and possibly death (4). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal)

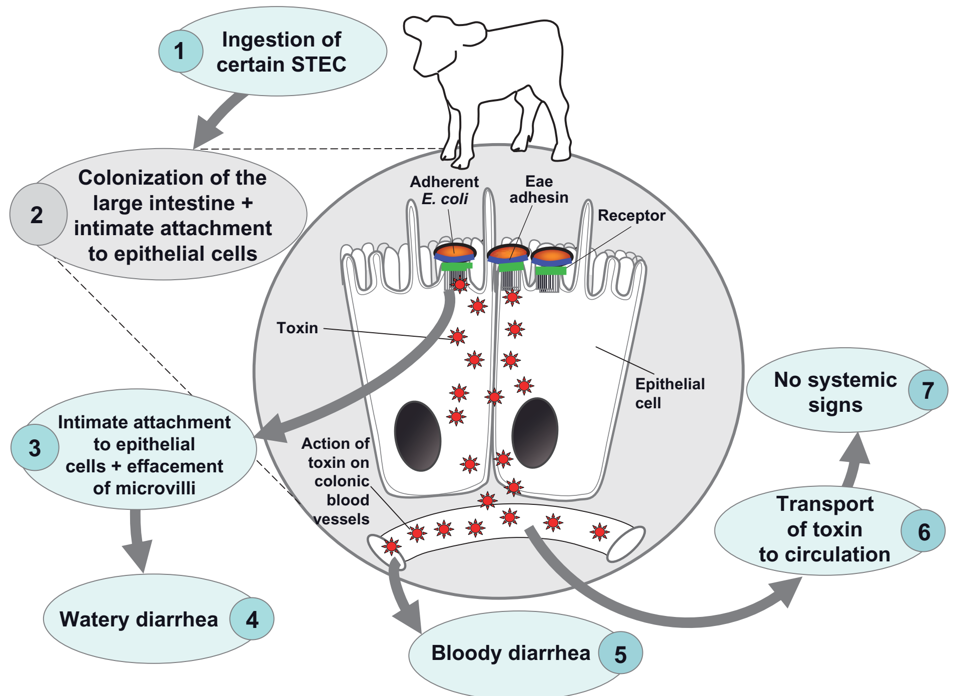

Figure 15.7. Schematic representation of the steps involved in the pathogenesis of STEC infection in dysentery of calves. STEC are ingested, pass through the stomach, and colonize the large intestine through formation of attaching and effacing lesions (1–3). Diarrhea may result from loss of absorptive microvillus surface, activation of secretory activity in epithelial cells, and loosening of tight junctions (4). Shiga toxin 1 and/or 2 is produced in the intestine, and absorbed into the blood; bloody diarrhea is presumed to be due to the action of Shiga toxin locally (5). No systemic signs are observed due to inactivation of the Stx (6). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal)

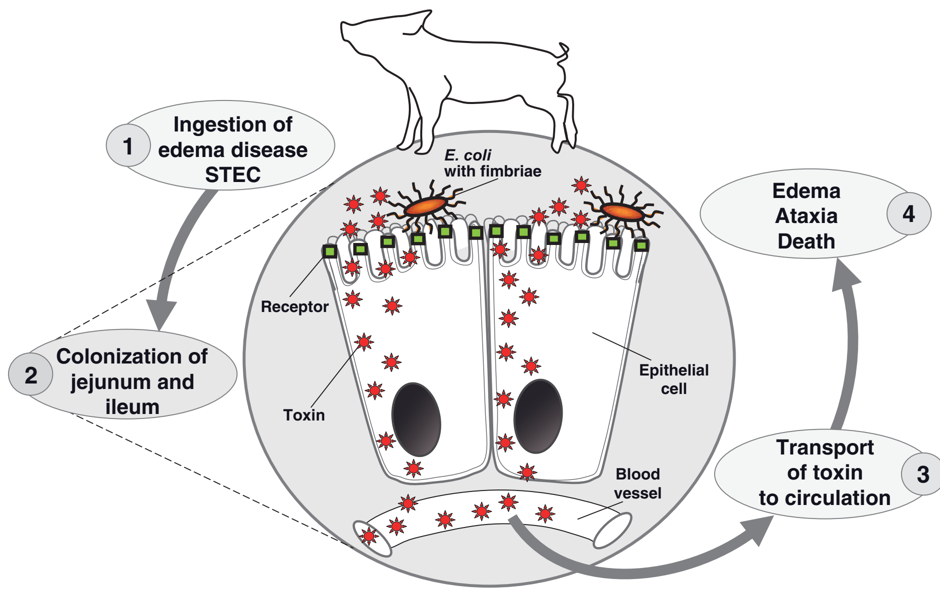

Figure 15.8. Schematic representation of the steps involved in the pathogenesis of STEC infection in edema disease of pigs. The initial steps (1, 2) of ingestion of bacteria and colonization of the intestine are similar to those for ETEC. Shiga toxin 2e (Stx2e) is produced in the intestine and absorbed into the blood (3). The Stx2e binds to receptors on vascular endothelium in the central nervous system and other sites including the stomach and subcutaneous tissues of the forehead and eyelids, giving rise to edema, ataxia, and death (4). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal)

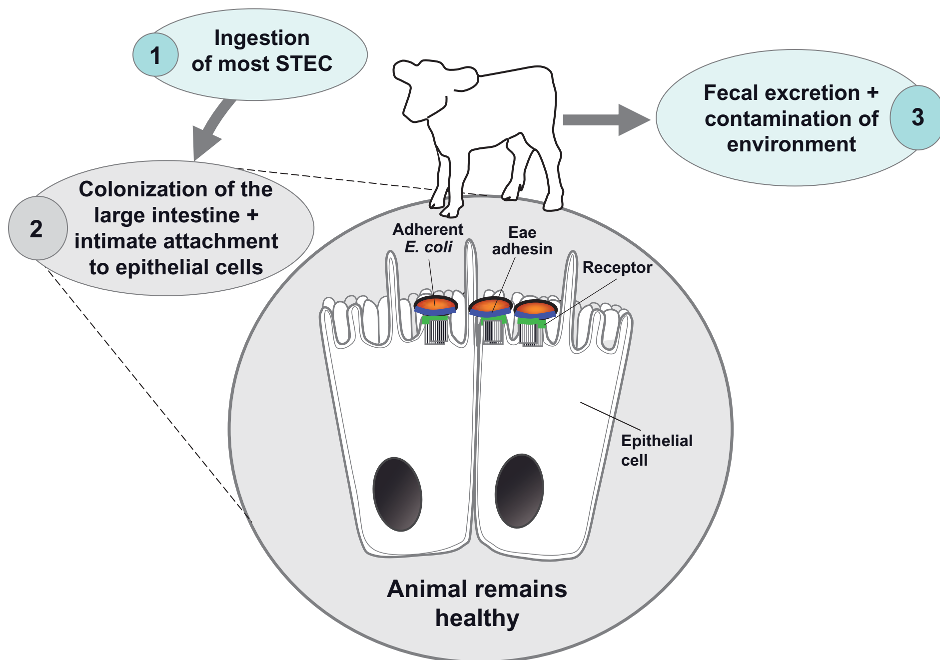

Figure 15.9. Schematic representation of the steps involved in the colonization of the intestine in cattle and sheep and other ruminants by STEC that are pathogenic for humans. These STEC are ingested, pass through the stomach, and colonize the large intestine through formation of attaching and effacing lesions or possibly other adhesins (1, 2). These STEC do not cause disease in natural conditions but may be shed in the feces for long periods and contaminate the environment (3), including food and water consumed by humans.

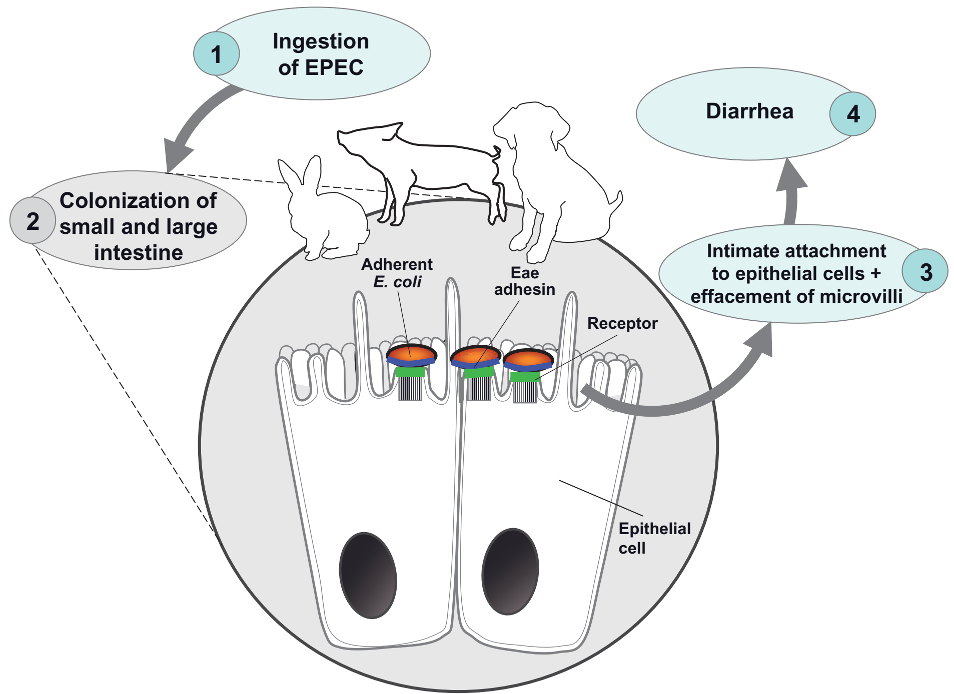

Figure 15.10. Schematic representation of the steps involved in the pathogenesis of EPEC infection in pigs, rabbits, dogs, and other animal species. EPEC are ingested, pass through the stomach, and colonize the small and large intestine through formation of attaching and effacing lesions (1–3). Diarrhea results from loss of absorptive microvillus surface, activation of secretory activity in epithelial cells, and loosening of tight junctions (4). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal)

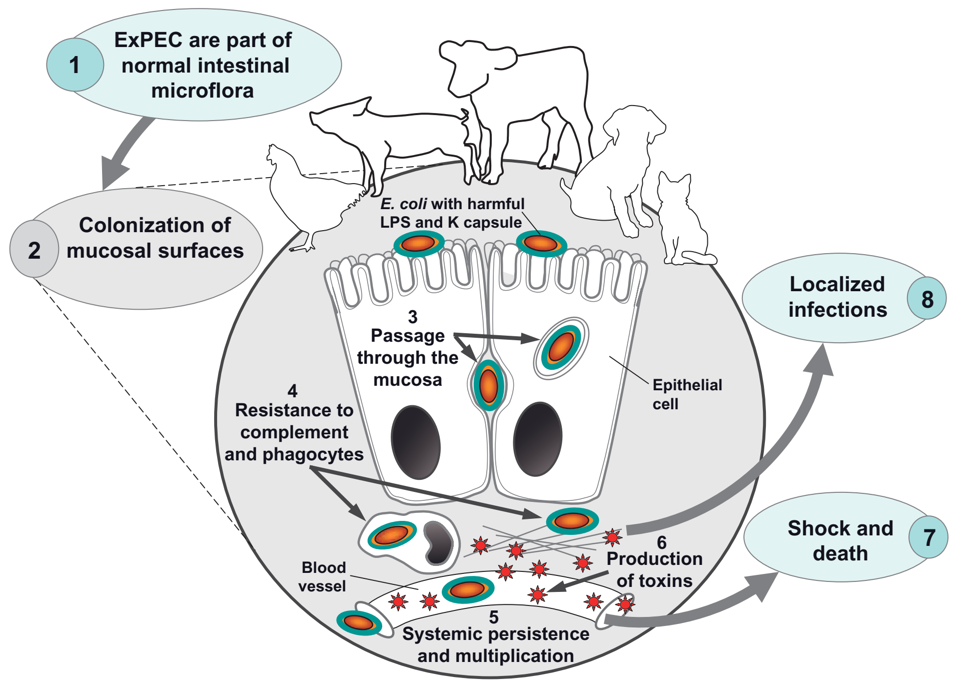

Figure 15.12. Schematic representation of the steps involved in the pathogenesis of ExPEC infection. Ingested bacteria colonize the intestine (1, 2) then pass through and between epithelial cells to gain access to the underlying tissue (3). Resistance to complement and phagocytes permits survival of the bacteria (4) which are transported to distant organs via the blood stream (5). Production of toxins, notably LPS, locally and in the blood stream lead to localized and generalized disease (7, 8). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal)

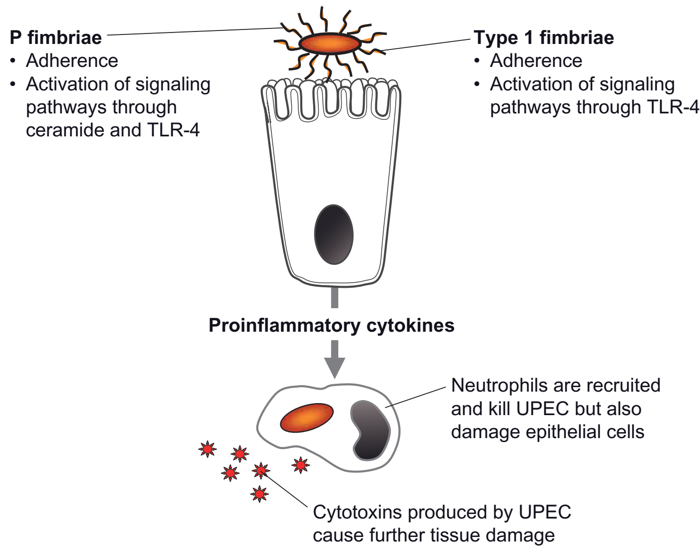

Figure 15.13. Uropathogenic *E. coli* (UPEC) in cystitis. UPEC may adhere through binding of the FimH adhesin of type 1 fimbriae to mannosylated glycoproteins and to TLR-4 (coreceptor) on the surface of uroepithelial cells and activate TLR-4. P fimbriae bind to Galα(1–4)Galβ receptors in ceramide-anchored glycosphingolipids, resulting in the release of ceramide which activates TLR-4. Activation of the epithelial cells results in secretion of cytokines, notably IL-6 and IL-8, which recruit neutrophils to the area. The neutrophils engulf and kill the UPEC.

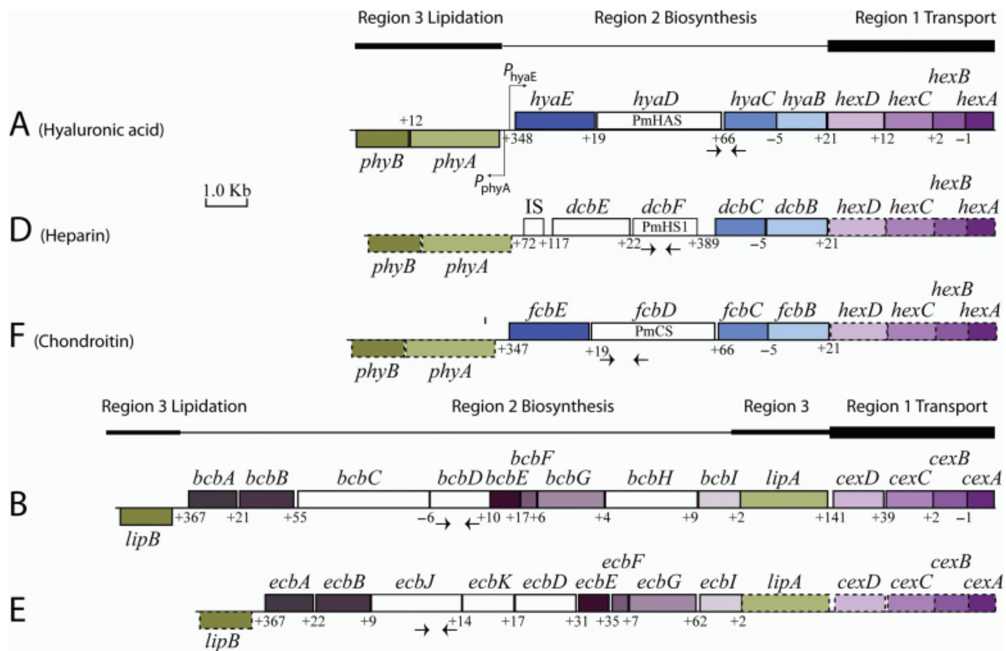

Figure 17.1. Genetic organization of the capsule biosynthetic loci of representative isolates of the *P. multocida* serogroups A, D, F, B, and E. Sequences shown were determined from the strains X-73 (A), P934 (D), P4218 (F), M1404 (B), and P1234 (E). Boxes above the lines indicate genes transcribed from left to right while boxes below the lines represent genes transcribed from right to left. Dotted boxes indicate genes that have not had their sequences completely determined but have been shown by PCR and restriction fragment length analysis to be highly similar to the corresponding sequenced strains. Gene names are shown either above or below the corresponding boxes and the names of the experimentally proven synthases are shown within the corresponding boxes. Small arrows (not to scale) indicate the positions of primers used for capsular PCR typing (Townsend et al. 2001). The right-angled arrows between *phyA* and *hyaE* in the type A diagram indicate the position of the two predicted *cap* locus promoters. The grouping of genes into regions associated with transport, biosynthesis, or lipidation is shown above the type A diagram (for serotypes A, D, and F) and above the type B diagram (for serotypes B and E). Genes encoding proteins with >50% identity are shaded identically. Numbers below or above the box indicate the distances between genes in bp (modified from Townsend et al. 2001).

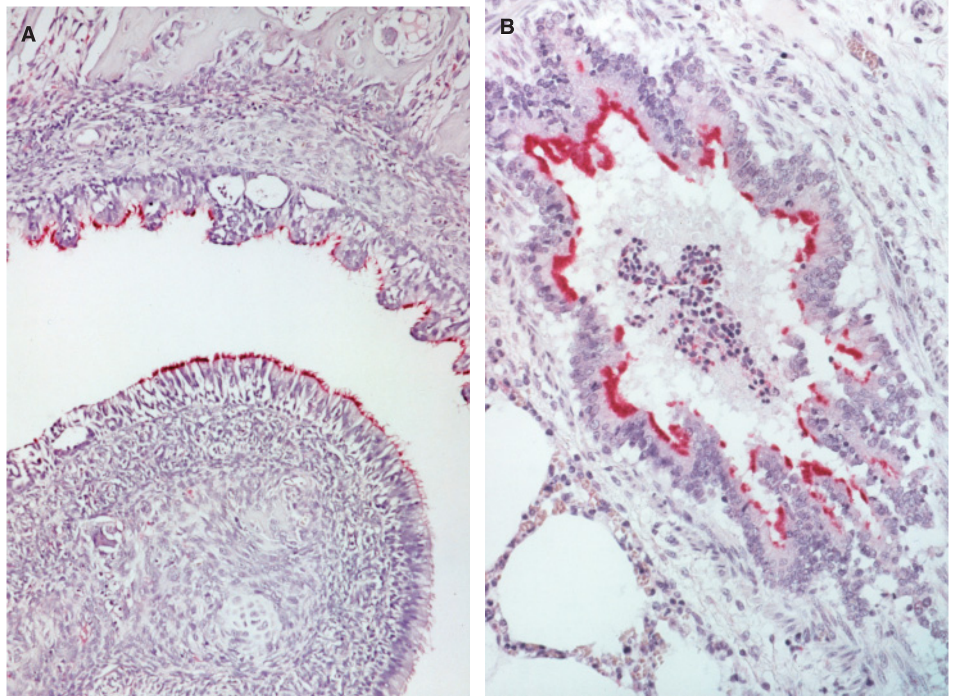

Figure 21.1. Colonization by *B. bronchiseptica* (red staining) of the ciliated epithelium of the conchae (A; 160×) or bronchus (B; 400×) of an infected pig. Areas denuded of cilia are apparent in the conchae. A neutrophilic inflammatory infiltrate is present in the lumen of the bronchus.

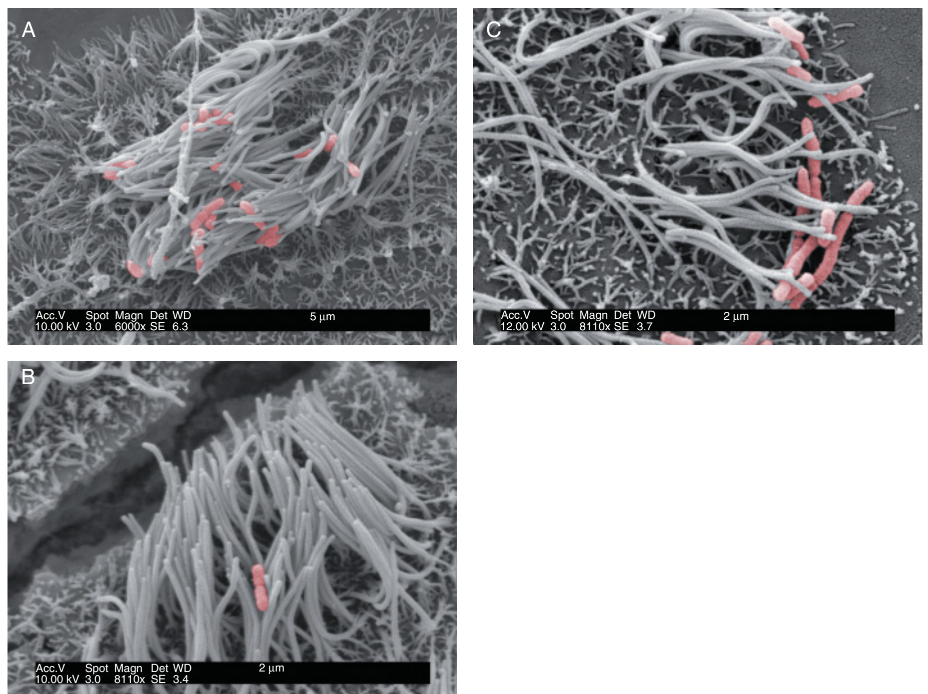

Figure 21.2. Binding of *B. bronchiseptica* to ciliated tracheal epithelial cells visualized by scanning electron microscopy. (A) Maximal binding, with preferential attachment to cilia, occurs when bacteria are in the fully modulated Bvg$^+$ phase. (B) A mutant strain locked permanently in the Bvg$^-$ phase displays a greatly diminished capacity for adherence. (C) Ectopic expression of FHA by a phase-locked Bvg$^-$ mutant restores ciliary attachment.

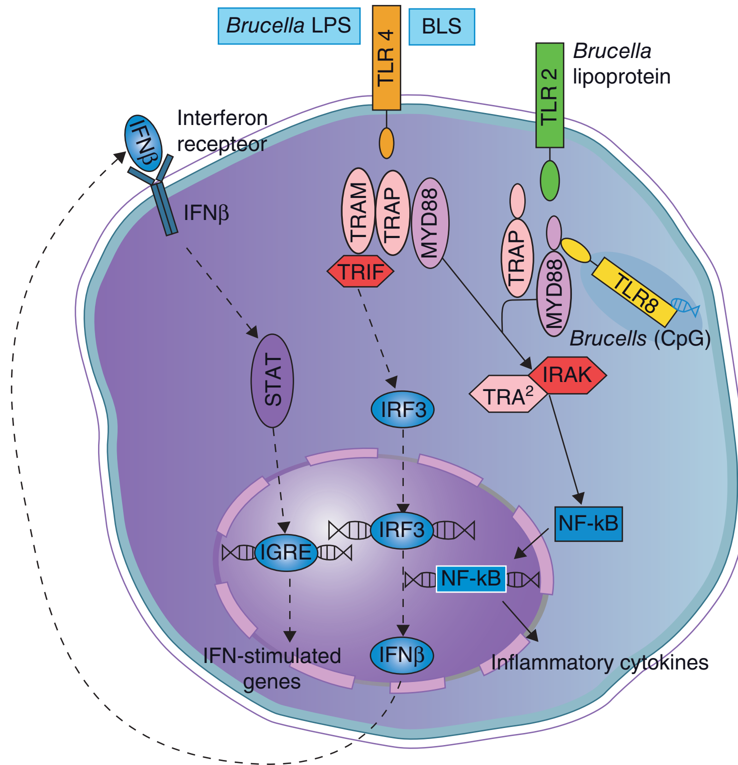

Figure 22.1. TLRs and signaling pathways through which *Brucella* can interact.

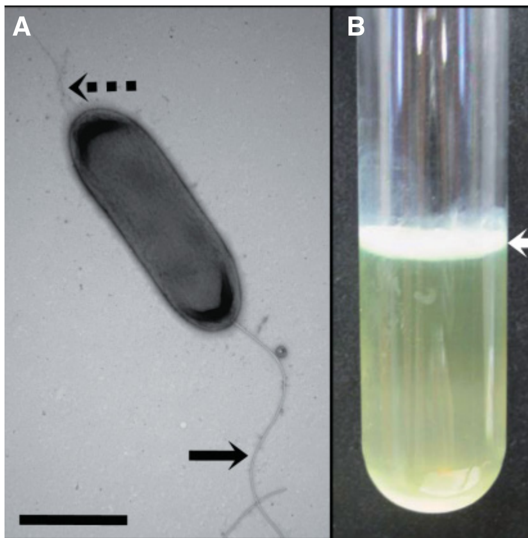

Figure 23.1. *P. aeruginosa.* (A) Transmission electron micrograph of negatively stained *P. aeruginosa* cells. Black arrow points to the single polar flagellum, and the dashed arrow indicates pili. Bar: 1 µm. (B) Stationary-phase culture normally exhibits the characteristic blue-green color due to siderophore secretion. The white arrow points to the pellicle, a biofilm at the liquid/air interface.

15
Escherichia coli

C. L. Gyles and J. M. Fairbrother

INTRODUCTION

Escherichia coli is a gram-negative, fermentative, rod-shaped bacterium which grows readily on simple bacteriologic media, including MacConkey agar, on which it forms large red colonies. Other features that are helpful in its identification include a positive indole reaction, negative tests for production of urease and hydrogen sulfide, and failure to utilize citrate as the sole carbon source. Although vastly outnumbered by anaerobic bacteria, *E. coli* is the major facultatively anaerobic bacterium in the intestinal tract of most animal species and is typically present at 10^7–10^9 organisms per gram in feces. *E. coli* is usually the dominant organism recovered on aerobic culture of feces, but pet birds are an exception as *E. coli* are recovered from only a low percentage of healthy pet birds.

The sterile intestinal tract of the newborn animal quickly becomes contaminated with bacteria, including *E. coli*, from the microflora of the dam and from the environment. *E. coli* rapidly becomes established in the intestine and remains as a part of the normal flora throughout the life of the animal. The concentration of *E. coli* is low in the upper small intestine but increases progressively, with the maximum concentration in the large intestine. Most *E. coli* are commensals, that is, they reside in the intestine but are not harmful for the host animal. Only a small proportion of strains are pathogenic, being classified into categories or pathotypes based on the production of broad classes of virulence factors and on the mechanisms by which they cause disease. In animals, the most important pathotypes are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin (Stx)-producing *E. coli* (STEC), and extraintestinal pathogenic *E. coli* (ExPEC). In addition, STEC in the normal flora of cattle and other ruminants may be highly pathogenic for humans. Also, ExPEC and EPEC typically are carried as part of the normal intestinal flora of their host and may be considered as opportunistic pathogens. Collectively, the set of *E. coli* which are pathogenic in animals or causative of zoonotic diseases in humans may be referred to as animal pathogenic and zoonotic *E. coli* (APZEC).

In most *E. coli* diseases, pathogenicity is associated with virulence genes encoded by plasmids, bacteriophages, or pathogenicity islands (PAIs). These genes include the plasmid-encoded genes for enterotoxins and fimbriae or pili, the phage-encoded genes for Stx, and the PAI-encoded genes for the attaching and effacing (AE) lesion in EPEC and enterohemorrhagic *E. coli* (EHEC) as well as the *pap*, *hly*, and *cnf1* genes in uropathogenic *E. coli* (UPEC), which are subsets of the STEC and ExPEC pathotypes, respectively.

Differentiation of *E. coli* into subtypes is important for distinguishing pathogenic from nonpathogenic types and for epidemiological investigations. Serotyping is a well-established method that is based on differences in the O, K, and H antigens determined by the polysaccharide portion of lipopolysaccharide (LPS), capsular polysaccharide, and flagellar proteins, respectively (Scheutz et al. 2004). The K antigens are no longer routinely determined and serotyping usually involves determination of O and H antigens. Presently, there are 174 O antigens (O1–O181, with O groups 31, 47, 67, 72, 93, 94, and 122 removed) and 53 H antigens (H1–H56, with

13, 22, and 50 unassigned) in the international typing scheme. F or fimbrial antigens are additional surface antigens, whose identification provides valuable information in the characterization of strains. The F antigen is therefore often added to the serological formula for a strain, especially for ETEC of animal origin. Serotyping remains the foundation of subspecies differentiation. Complete serotyping is conducted by only a few laboratories but many clinical laboratories identify a subset of O antigens. Increasingly, polymerase chain reaction (PCR) methods are being developed for detection of the genes that are specific for several important O and H antigens (Prager et al. 2003).

Other procedures that are used to characterize isolates include pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, amplified fragment length polymorphism (AFLP), ribotyping, 16S rDNA sequencing, multilocus sequence typing (MLST), multiple-locus variable-number tandem-repeat analysis (MLVA), and virulence factor analysis. Biotyping on the basis of a selection of biochemical tests is also useful but has only moderate discriminatory power.

There are at least 28 colonization and fitness factors and 33 toxins and effector molecules in pathogenic *E. coli* (Kaper et al. 2004). Detection of virulence factors that are unique to or associated with particular types of pathogenic *E. coli* is important for identification and characterization of pathogenic *E. coli*. As mentioned above, the term pathotype, or less commonly pathovar, is used to identify types of *E. coli* on the basis of their virulence mechanism (Levine 1987; Nataro and Kaper 1998; Milon et al. 1999) as indicated by the presence of virulence genes which characterize the process by which disease is caused. This system identifies broad classes of pathogenic *E. coli*, such as ETEC, EPEC, EHEC, ExPEC, and UPEC. The term virotype will be used to refer to variants within these classes, based on differences in the complement of virulence genes. Thus, the virotyping system identifies ETEC variants based on the complement of genes for fimbriae and enterotoxins among ETEC.

TYPES OF *ESCHERICHIA COLI* IMPLICATED IN DISEASE

Escherichia coli cause a wide variety of enteric and extraintestinal diseases in animals (tables 15.1 and 2). These diseases have been studied extensively in food-producing animals but only to a limited extent in other species. For example, there is little information on *E. coli* infections in horses, in which *E. coli* does not appear to be a significant pathogen.

ETEC

ETEC is the most common cause of *E. coli* diarrhea in farm animals (Fairbrother et al. 2002; Nagy and Fekete 2005). This pathotype is characterized by the production of enterotoxins and of adhesins that adhere to the intestinal epithelium and promote colonization. Enterotoxins produced by ETEC may be heat stable (STa, STb, or enteroaggregative *E. coli* heat-stable enterotoxin 1 [EAST1]) or heat labile (LT). In pigs, the most frequently encountered fimbrial adhesins of ETEC are K88 (F4), K99 (F5), 987P (F6), F41, and F18. A nonfimbrial adhesin (adhesin involved in diffuse adherence [AIDA-I]) has recently been associated with certain ETEC from pigs. Isolates producing the K88 (F4) or F18 adhesin and certain isolates producing 987P (F6) are hemolytic. In calves and lambs, the most important fimbrial adhesins of ETEC are K99 (F5) and F41. Colonies of ETEC that possess these adhesins are usually mucoid and nonhemolytic. F17 fimbriae are associated with bovine or ovine strains that cause diarrhea or septicemia. The most important virotypes are listed in tables 15.3–5.

Virulence Factors of ETEC

ETEC Fimbrial and Afimbrial Adhesins

Fimbriae (or pili) are rodlike or fibrillar surface appendages on bacteria which mediate attachment to host tissues. Each fimbrial unit consists of hundreds of copies of a major subunit which provides the structure and confers the antigenic specificity of the fimbriae. Fimbriae may also contain several copies of minor subunits, one of which may be an adhesin with specific binding properties. The nomenclature of fimbriae is not standardized and fimbriae have been named based on such criteria as strain of origin (F41, 987P, F107), assumed structure (K88, K99), and putative function (colonizing factor antigen [CFA]). A system of F numbers was devised to designate fimbrial adhesins in the same way as the O and K antigens, and both this nomenclature and the original designations are used in the literature. Hence, the fimbriae originally named K88, K99, 987P, Fy, and F107, are synonymous with F4, F5, F6, F17, and F18. *E. coli* that possess enterotoxin genes but lack genes for the recognized

Table 15.1. Major Types of *E. coli* Implicated in Enteric Diseases of Animals

Animal species	Disease	Type of *E. coli*
Cattle	Neonatal diarrhea	STa$^+$, K99$^+$, F41$^+$ ETEC
	Diarrhea/dysentery in 2–8-week-old calves	STEC of a wide variety of O groups, notably 5, 26, 111, 118
Pigs	Neonatal diarrhea	STa$^+$, K99$^+$,F41$^+$ ETEC
		LT$^+$, STb$^+$ O149:K88ac ETEC
		LT$^+$, STa$^+$, STb$^+$ O149:K88ac ETEC
	Postweaning diarrhea	LT$^+$, STb$^+$ O149:K88ac ETEC
		LT$^+$, STa$^+$, STb$^+$ O149:K88ac ETEC
		F18$^+$ O138 ETEC
		F18$^+$ O141 ETEC
		EPEC of O groups 45, 103
	Edema disease	F18$^+$ Stx2e$^+$ O139 STEC
		F18$^+$ Stx2e$^+$ O138 ETEC/STEC
		F18$^+$ Stx2e$^+$ O141 ETEC/STEC
Dogs	Diarrhea	ETEC, primarily STa$^+$
		EPEC
Rabbits	Diarrhea	EPEC of O groups 2, 15, 103, 128, 132

Table 15.2. Extraintestinal Diseases of Animals Caused by *E. coli*

Host species	Type of disease	Comments
Cattle	Septicemia	Invasive *E. coli* that affect calves with hypogammaglobulinemia; O groups 11, 78, 101, 115, 119, NT.
	Mastitis	Environmental *E. coli* that act as opportunists.
Pigs	Septicemia	Invasion by specific types of *E. coli* in pigs that are deficient in immunity.
	Mastitis	Opportunistic infection of nursing sows.
Dogs	UTI	Most commonly O4 or O6 *E. coli* that are hemolytic, papG (allele III)$^+$, *cnf1*$^+$.
	Pyometra	The same types of *E. coli* that cause urinary tract infection.
	Septicemia	May occur as a sequel to parvovirus infection; may be seen in young puppies.
Cats	UTI, pyometra, septicemia	The *E. coli* have similar properties to those isolated from dogs.
Poultry	Air sacculitis/ septicemia	*E. coli* especially of O groups 1, 2, and 78 are implicated in a wide range of infections of poultry; predisposing factors are usually necessary for disease.
	Cellulitis	Same as in air sacculitis/septicemia.
	Yolk sac infection	
	Swollen head syndrome	

UTI—urinary tract infection.

Table 15.3. Important Pathotypes, Virotypes, and Serogroups of *E. coli* Causing Disease in Pigs

Disease	Pathotype	Virotypes	O serogroups
Enteric			
Neonatal diarrhea	ETEC	STa:K99:F41, STa:F41, STa:987P, LT:STb:EAST1:K88ac, LT:STb:STa:EAST1:K88ac, STb:EAST1:AIDA	8,9,20,45,64,101,138,141, 147,149,157
Postweaning diarrhea	ETEC	LT:STb:EAST1:K88ac, LT:STb:STa:EAST1:K88ac, STa:STb, STa:STb:F18ac, STa:F18ac	8,138,139,141,147,149, 157
	EPEC	Eae, Tir, EspA, EspB, EspD, EspC (enterotoxin)	45,103,123
Edema disease	STEC	Stx2e:F18ab:(AIDA), _Hly^{+}	138,139,141
Extraintestinal			
Colisepticemia	SEPEC	Aerobactin, F165-1 (P fimbrial family), F165-2 (S fimbrial family), CNF1 or CNF2, CDT	6,8,9,11,15,17,18,20,45, 60,78,83,93,101,112, 115,116
Urogenital tract infection	UPEC	P, S, aerobactin, CNF1	1,4,6,18

Table 15.4. Important Pathotypes, Virotypes, and Serogroups of *E. coli* Causing Disease in Cattle and Sheep

Disease	Pathotype	Virotypes	O serogroups
Enteric			
Neonatal diarrhea	ETEC	STa:K99:F41, STa:F41	8,9,20,64,101
Dysentery	STEC	Eae:Stx1 and/or Stx2	5,8,20,26,103,111,118,145
Extraintestinal			
Septicemia	SEPEC	P:CNF1 F17: CNF2: CDT	8,9,15,26,35,45,78,86,101,115,O117,137
Mastitis		LPS	Diverse

Table 15.5. Important Categories, Virulence Factors, and Serogroups of *E. coli* Causing Disease in Poultry

Disease	Category	Virulence factors	O serogroups
Septicemia	APEC	F1 (type 1), F11 (P fimbrial family), Sit, Stg K1, aerobactin, salmochelin, Tsh,	1,2,8,15,18,35,78,88,109,115
Cellulitis	APEC	F1- and P-fimbriae, K1	2,25,71,78

fimbriae have been isolated from diarrheic animals. This is illustrated by a recent report from North Vietnam, where O8 serogroup hemolytic *E. coli* from preweaned pigs with diarrhea had genes for LT, STa, and STb but were negative for F4, F5, F6, F18, and F41 fimbriae (Do et al. 2006a). These O8 isolates produced fimbriae *in vitro* and colonized the ileum and caused acute watery diarrhea in experimental infection of 1-day-old colostrum-deprived pigs (Do et al. 2006b). These findings indicate that there are uncharacterized adhesins that play a role in ETEC infections in pigs.

K88 (F4) Fimbriae. The K88 (F4) fimbria, initially thought to be a capsular antigen, was later determined to be a fine fibrillar structure. The major subunit of the K88 (F4) fimbriae constitutes the adhesin. K88 (F4) fimbriae are encoded by the *fae* locus on a plasmid which often also carries genes for raffinose fermentation. The O serogroups and virotypes most commonly associated with K88 (F4) fimbriae are listed in table 15.3. K88 fimbriae mediate bacterial adherence to the intestinal epithelium throughout most of the small intestine of pigs of all ages. Hence, colonization of the intestinal mucosa by K88-positive ETEC occurs in both neonatal and postweaning pigs and may be observed in finisher pigs.

Adherence due to K88 (F4) fimbriae is species specific, occurring almost exclusively in pigs. There are three variants of K88, namely, ab, ac, and ad, with the ac variant being most common. Some pigs are resistant to colonization by K88-positive ETEC because they lack the receptors for the K88 adhesin that are found on chromosome 13. The allele (S) for the K88 (F4) receptor is dominant and genetic resistance to adherence by K88-positive ETEC is inherited in a Mendelian fashion. Hence, there are three genotypes: ss (resistant), SS and Ss (sensitive). A PCR test that detects resistance/susceptibility of pigs to intestinal adherence of K88-positive ETEC has been developed. If the sow is the resistant parent, there is no specific anti-K88 antibody in the colostrum in the absence of parenteral immunization, resulting in highly susceptible piglets. The biologically relevant K88 (F4) receptor has been identified as an intestinal brush border mucin-type sialoglycoprotein (IMTGP) and susceptibility of pigs to K88-positive ETEC may be determined by *in vitro* tests of adherence of the bacteria to brush borders or enterocytes (Francis et al. 1998; Francis 2002). The peptide that includes amino acids 125–163 of FaeG appears to be essential for K88 variant-specific binding (Zhang et al. 2009).

Certain proteins in porcine milk bind K88 fimbriae and may thereby contribute to host defense against K88-positive ETEC (Shahriar et al. 2006). These proteins include lactadherin, lipoprotein lipase, β casein, and whey acidic protein. Lactadherin blocked the attachment of K88ac-positive ETEC to pig intestinal villi *in vitro*.

K99 (F5) Fimbriae. K99 (F5) fimbriae were also initially thought to be capsular, but were subsequently shown to be fibrillar appendages. They are encoded by the *fan* locus which is found on a plasmid. As with K88 fimbriae, the major fimbrial subunit (FanC) is the adhesin, allowing the fimbriae to bind laterally rather than by the tip. K99 fimbriae are produced by ETEC from pigs, cattle, and sheep (tables 15.3 and 4). K99 (F5) fimbriae bind to the specific receptor N-glycolylneuraminic acid-GM3 and mediate attachment of ETEC mostly to the posterior small intestinal mucosa in younger, and occasionally, in older animals. Hence, diarrhea due to K99-positive ETEC is observed mostly in neonatal pigs, calves, and lambs.

987P (F6) Fimbriae. 987P (F6) are large rod-shaped fimbriae, encoded by the *fas* locus which is found on the chromosome and on plasmids and encodes three structural proteins, the major subunit FasA and minor subunits FasF and FasG. FasG is the adhesin. F6 mediates bacterial colonization mostly of the posterior small intestine in neonatal pigs. F6-mediated intestinal colonization in older pigs is rarely observed and is thought to be inhibited by preferential binding of bacteria to F6 receptors present in the intestinal mucus of older pigs rather than to receptors on the intestinal epithelium. Hence, diarrhea due to 987P-positive ETEC is observed almost exclusively in neonatal pigs. ETEC with this fimbria have been reported at low frequency in recent years.

Three specific receptors have been reported for 987P (F6) fimbriae. The adhesin, FasG, binds both hydroxylated sulfatide and histone H1 on intestinal epithelial cells and the major subunit, FasA, binds hydroxylated ceramide monohexoside.

F18 Fimbriae. F18 are long flexible filaments with a characteristic zig-zag pattern (Nagy and Fekete

1999) that are encoded by the *fed* locus which is usually found on a plasmid. Binding is mediated by the adhesin FedF. There are two variants of F18, ab and ac, based on serological and nucleotide sequence differences. These variants are biologically distinct, F18ab being poorly expressed *in vitro* and *in vivo* and most often associated with Stx2e-producing strains of *E. coli* implicated in edema disease (ED), and F18ac being more efficiently expressed *in vitro* and *in vivo* and more commonly associated with ETEC strains. Receptors for F18 are lacking in newborn pigs and are increasingly produced up to weaning age; they are retained in adult pigs. Genotypes susceptible and resistant to F18 adherence have been differentiated, and pigs with at least one copy of the dominant allele for the receptor are susceptible to epithelial cell adherence, and hence to intestinal colonization. The receptor gene has been localized on porcine chromosome 6, closely linked to the gene encoding halothane sensitivity, but there appears to be no coselection for F18 receptor and halothane sensitivity (Coddens et al. 2007). There is a high correlation between presence of the F18 receptor and the H-2 and A-2 histo-blood group antigens (HBGAs) and some evidence that there is a functional role for the A-2 HBGAs (Coddens et al. 2007).

A plasmid-encoded PAI which includes the *estB* gene has been identified in certain F18-positive ETEC and ETEC/STEC (Fekete et al. 2003).

F41 Fimbriae. F41 are fibrillar appendages encoded by genes present on the chromosome and are found on both bovine and porcine ETEC strains. They are mostly expressed together with K99 fimbriae on ETEC of O groups 9 and 101, although some strains, mostly from pigs, may produce F41 alone. F41-mediated colonization is observed in the posterior small intestine in neonatal pigs, calves, and lambs, and may result in diarrhea, whether K99 fimbriae are present or not.

F17 Fimbriae. F17 are flexible fimbriae found mostly on bovine necrotoxigenic *E. coli* (NTEC) strains producing cytotoxic necrotizing factor (CNF) 1 or CNF2, being encoded by genes on the chromosome in the former and on a plasmid in the latter (Mainil et al. 2000). Four gene variants, a, b, c, and d, have been described, based on differences in the major subunit F17A. F17 has also been found on bovine ETEC strains, although its role in diarrhea due to these strains has not been elucidated. F17 fimbriae consist of a major structural pilin (F17A) with a flexible tip fibrillum that contains the F17G adhesin. Like the adhesins of type 1 and P fimbriae, the lectin domain of F17G has an immunoglobulin-like fold. F17G has its binding site located laterally and has been proposed to insert between microvilli in order to bind (Buts et al. 2003).

AIDA-I, A Nonfimbrial Adhesin. The adhesin involved in diffuse adherence (AIDA-I) is an autotransported 100-kDa mature protein, which mediates bacterial attachment to intestinal epithelial cells. AIDA-I was originally detected in *E. coli* isolates from humans with diarrhea, and homologs have been detected subsequently in other *E. coli* including strains from pigs with ED or postweaning diarrhea, particularly in strains of the virotypes Stx2e:F18 and F18 alone (Mainil et al. 2002). A high percentage of the AIDA-positive porcine strains carry the *astA* gene for enteroaggregative heat-stable enterotoxin (Zhao et al. 2009). In these strains, AIDA is encoded by genes on a plasmid, possibly the same as that containing the *fed* genes that encode F18 fimbriae. ETEC isolates of the STb or STb:EAST-1 virotypes from neonatal or weaned pigs may also be AIDA-positive and induce diarrhea in neonatal pigs (Ngeleka et al. 2003). AIDA is also present in nonenterotoxigenic *E. coli*. AIDA-I was shown to be involved in biofilm formation and to be necessary for colonization of newborn pigs by an AIDA-I+, STb+ O143 isolate from a weaned pig with diarrhea (Ravi et al. 2007).

Enterotoxins

Two major classes of enterotoxin are produced by ETEC (Gyles 1994; Turner et al. 2006): low-molecular-weight heat-stable toxins (ST) which are resistant to 100°C for 15 min, and high-molecular-weight heat-labile toxin (LT), which is inactivated at 60°C for 15 min. Both types of enterotxin are plasmid-encoded. ST has been further characterized as STa (or STI) and STb (or STII) based on size, molecular structure, and biological activity. The gene for EAST1, which is related to STa, is present in a wide variety of *E. coli*, including porcine ETEC. LTs are highly antigenic, whereas STs are poorly antigenic. Enterotoxins cause disturbances in intestinal fluid metabolism but do not produce pathological lesions or morphological changes in the intestinal mucosa.

STa. STa (also called STI) is an 18- or 19-amino acid peptide of about 2000 Da, which is produced as a pre-pro-peptide, transported across the inner membrane, folded in the periplasm, then secreted through TolC. STa has been designated STaP (produced by bovine, porcine, and human ETEC) or STaH (produced by human ETEC), based on minor differences in composition. All forms of STa have a conserved C-terminal 13 amino acids which include three disulfide bonds. STa binds to a guanylyl cyclase C (GC-C) glycoprotein receptor on the brush border of villous and crypt intestinal epithelial cells and activates guanylate cyclase, which stimulates production of cyclic GMP (cGMP) (Giannella and Mann 2003; Turner et al. 2006; Al-Majali et al. 2007; fig. 15.1). Elevated levels of cGMP in the cell activate cGMP-dependent protein kinase II (cGKII) resulting in phosphorylation of the chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR). Activation of CFTR results in secretion of Cl^- and HCO_3^- as well as inhibition of absorption of Na^+ (fig. 15.1). Based on the concentration and affinity of the STa receptors, the posterior jejunum appears to be the major site of hypersecretion in response to STa. There is good evidence that STa binds to other receptors (Sellers et al. 2008) and that it has other activities but their relationship to diarrhea is not known. Interestingly, STa and analogues inhibit proliferation of colon cancer cells and it may have a future as an antiangiogenic and antimetastatic molecule.

The effects of STa are reversible. STa is active in infant mice and young pigs but is less active in older pigs, consistent with decreasing affinity and density of STa receptors with increasing age. Not surprisingly, ETEC strains that produce STa as the only enterotoxin are associated with disease in neonatal pigs, calves, and lambs.

The gene that encodes STa, *estA*, has an AT content of 70% and is associated with transposons that are carried on plasmids.

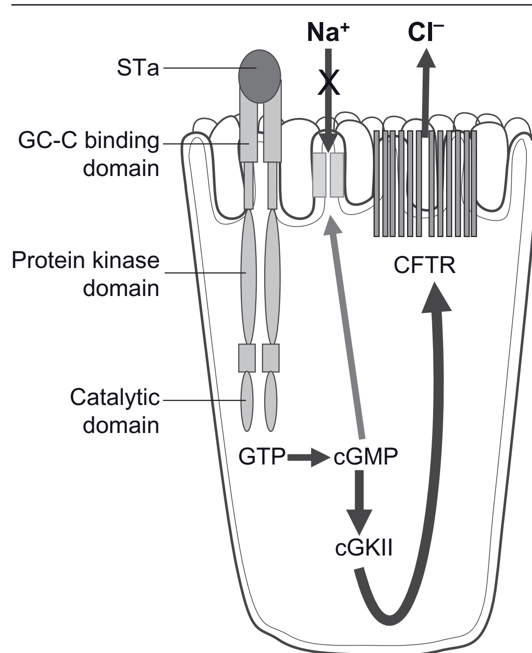

Figure 15.1. Mechanism of action of STa. Binding of STa to its receptor (GC-C) activates the catalytic domain, causing conversion of GTP to cGMP, which activates the cGMP-dependent kinase cGKII. This kinase phosphorylates CFTR, causing secretion of Cl^- and HCO_3^- and inhibition of absorption of Na. (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal)

STb. STb (also called STII) is a 48-amino acid peptide of 5 kDa that is unrelated to STa in composition and mechanism of action (Dubreuil 1997, 2008). STb is synthesized as a precursor that is released into the periplasm where it is converted to an active form with two intramolecular disulfide bonds. It is exported across the outer membrane through TolC and accessory proteins. ETEC producing STb are mostly associated with pigs and the majority of porcine ETEC produce STb. A variant STb, with a change from histidine to asparagine at position 12 has been reported (Fekete et al. 2003; Taillon et al. 2008). This variant is encoded on a plasmid which also encodes STa and tetracycline resistance (*tetB*) and is found primarily on K88-negative ETEC. Differences in biological activity of the variant have not been reported.

The intestinal epithelial cell receptor to which STb binds has been identified as sulfatide (3′-sulfogalactosyl-ceramide) (Beausoleil et al. 1999; Gonçalves et al. 2008).

STb does not alter cGMP or cAMP levels in intestinal mucosal cells, thus differing in mechanism of action from STa and LT-I. Binding of STb to its receptor leads to uptake of Ca^{2+} into the cell, activating protein kinase C, which activates the CFTR (fig. 15.2). Elevated Ca^{2+} levels also stimulate synthesis

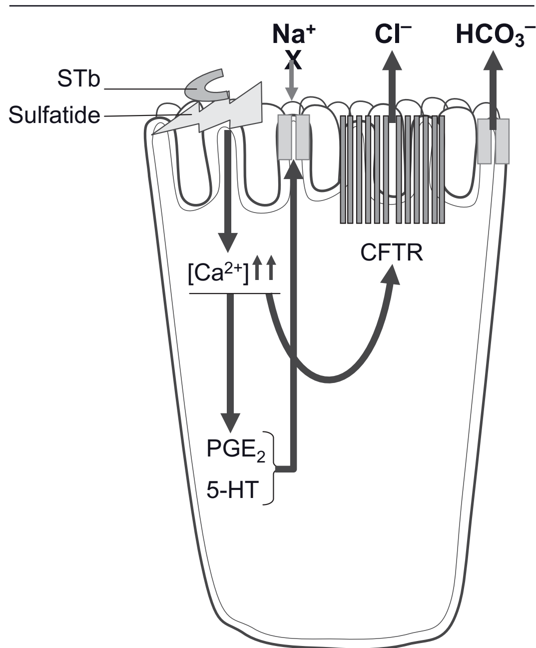

Figure 15.2. Mechanism of action of STb. Binding of STb to its receptor sulfatide leads to elevated intracellular levels of calcium. The high calcium levels lead to activation of the cystic fibrosis transmembrane conductance regulator (CFTR) and the secretagogues prostaglandin E_2 (PGE2) and 5-hydroxytryptamine (5-HT). These lead to secretion of Cl^- and HCO_3^- and inhibition of absorption of Na^+. (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal) (See color plate)

of secretagogues prostaglandin E_2 and 5-hydroxytryptamine, inducing the duodenal and jejunal secretion of water and electrolytes by unknown mechanisms (Harville and Dreyfus 1995).

STb permeabilizes intestinal epithelial cells *in vitro*, without killing them. *In vivo*, STb causes loss of villus epithelial cells and some villus atrophy. STb is inactivated by trypsin, and in the presence of trypsin-inactivator, is active in the intestines of mice, rats, and calves. Although ETEC producing only STb can induce diarrhea in young pigs (Fairbrother et al. 1989), its association with post-weaning pigs and experimental data (Erume et al. 2008) indicate that STb is likely to be of primary importance in older animals.

The estB gene which encodes STb is carried on plasmids.

EAST-1. EAST-1 was first identified in enteroaggregative *E. coli* isolated from humans. Subsequently, the EAST-1 gene (*astA*) has been detected in human, bovine and porcine ETEC, EPEC, and STEC. The *astA* gene is also present in *E. coli* with no other virulence genes. The *astA* gene is carried on a wide range of *E. coli* serotypes but appears to be almost always present in O149 ETEC, a major serogroup of porcine ETEC.

The *astA* gene is commonly found on plasmids in K88-positive ETEC strains from pigs with diarrhea and in F18:Stx2e strains from pigs with ED (Choi et al. 2001; table 15.1). EAST-1 is a 38-amino acid peptide of 4.1 kDa that has two di-sulfide bonds. It is different from STa and STb, although it shares 50% homology with the enterotoxic domain of STa (Veilleux and Dubreuil 2006) and appears to interact with the STa receptor guanylate cyclase C to elicit an increase in cGMP. Hence, the mechanism of action of EAST-1 is proposed to be identical to that of STa. However, the role of EAST-1 in the development of diarrhea has yet to be defined (Ménard and Dubreuil 2002).

Heat-Labile Enterotoxin (LT). Two subtypes of LT, LT-I, and LT-II, have been described, only LT-I being neutralized by anticholera toxin. LT-I can be divided into LT-Ih, produced by human ETEC, and LT-Ip, produced by porcine and human ETEC, that have slight differences in composition. LT-II consists of two antigenic variants, LT-IIa and LT-IIb, that are related to LTI in their A subunits but differ in their B subunits. Outside the host, the *eltAB* operon which encodes LTI is repressed by the binding of histone-like nucleoid structuring protein (H-NS) to a region downstream of the promoter. This H-NS repression is relieved under the conditions in the host intestine, temperature and osmotic pressure being two important factors.

LT-I is a high-molecular-weight toxin complex (~84 kDa) that consists of a biologically active A subunit and five B subunits that bind GM1 ganglioside receptors on the intestinal epithelial cell surface. Other receptors to which LT-I binds are GD1b, asialo GM1, GM2, and a number of galactoproteins and galactose-containing glycolipids. The A subunit (30 kDa) consists of an A1 fragment (21 kDa) containing the active site and an A2 fragment which links A1 to the B subunits. The A1 fragment appears to play an important role in stability of the holotoxin and differences in this fragment have been sug-

gested to account for the lower toxicity of LT-I compared with CT (Rodighiero et al. 1999). LT-I is very similar to cholera toxin (CT), both structurally and functionally. LT-I is transported across the outer membrane by a pathway highly homologous to the type II secretion pathway (T2SS) for secretion of CT (Tauschek et al. 2002). It binds LPS and becomes associated with outer membrane vesicles. Both LT-I and the T2SS are located at one pole of the bacterium and there is evidence that effective delivery of toxin involves intimate interaction of bacterium and host cell (Dorsey et al. 2006). After binding of the B subunits to their specific cell surface receptor, LT-I is internalized by receptor-mediated endocytosis then transported in a retrograde manner to the Golgi and the endoplasmic reticulum (ER) (fig. 15.3). After dissociation in the ER the A_1 fragment translocates into the cytoplasm and transfers an ADP-ribose moiety from NAD to the α subunit of the stimulatory regulator of adenyl cyclase located in the basolateral membrane. This permanently activates adenyl cyclase in the basolateral border of the cell. ADP-ribosylation is enhanced by ADP-ribosylation factors (ARFs), which are 20-kDa regulatory GTPases that activate the LT-I A1 catalytic unit.

High levels of cAMP in the cell activate the CFTR through phosphorylation by protein kinase A (Viswanathan et al. 2009). Opening of this anion channel results in increased secretion of Cl^- and HCO_3^- ions. There is also decreased absorption of Na^+ ions. Water follows by osmotic drag and diarrhea results. The effect of LT-I is irreversible and the affected enterocyte remains as a hypersecretor of cAMP until it is extruded. Excessive secretion of electrolytes and water leads to dehydration, metabolic acidosis, and possibly death. LT may also induce secretion by alternate mechanisms such as stimulation of prostaglandins, the enteric nervous system, and cytokine activation (Nataro and Kaper 1998).

LT-positive ETEC typically produce K88 fimbriae and STb, suggesting that there may be a functional connection between these virulence factors. Recent studies have shown that LT promotes adherence of ETEC *in vitro* and *in vivo* (Berberov et al. 2004; Johnson et al. 2009). Although the mechanism has not been identified it appears to require the ADP-ribosyl transferase activity of LT and does not involve an increase in K88 receptor on the enterocyte surface.

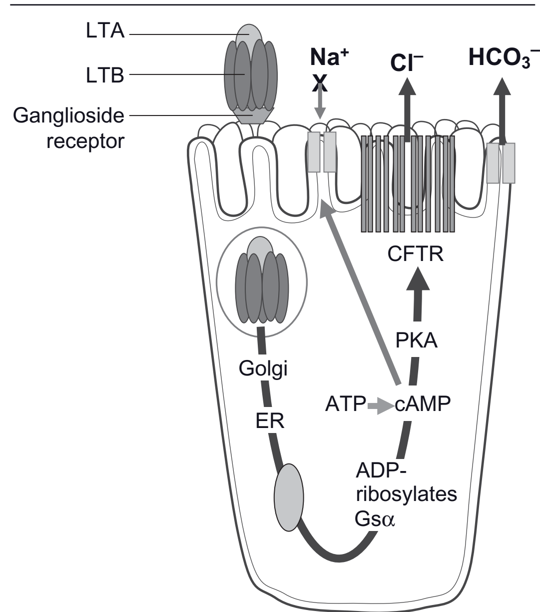

Figure 15.3. Major mechanism of action of LT. LT binds GM1 and other gangliosides, resulting in internalization by receptor-mediated endocytosis. Following transport to the Golgi and the ER, LTA1 is translocated to the cytosol where it ADP-ribosylates the regulatory protein Gsα which irreversibly stimulates production of cyclic AMP. Elevated levels of cAMP increase production of c-AMP-dependent protein kinase A, leading to phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR activation leads to secretion of Cl^- from secretory epithelial cells in the crypts, which stimulates HCO_3^- secretion from the Cl^-/HCO_3^- exchanger in villus epithelial cells (Sears and Kaper 1996; Viswanathan et al. 2009). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal) (See color plate)

LT has potent immunomodulatory effects and derivatives of LT are adjuvants of both the mucosal and systemic immune systems. LTB alone acts as an adjuvant by means of activation of cell signalling pathways following cellular uptake. Recent studies indicate that binding of LTB to GM1 alone is not likely to be solely responsible for triggering cell signaling and it is likely that binding to another receptor or a coreceptor might be involved (Fraser et al. 2003). It appears that the A and B subunits may have different immunomodulatory effects, the overall effect of the native LT being a result of the

combined effects of these subunits (Domingos et al. 2009). LT can also induce apoptosis of lymphoid cells, apparently by means of different mechanisms depending on the stage of differentiation of these cells (Tamayo et al. 2009).

There have been few studies of the occurrence of LTII-positive ETEC, but ETEC with the genes for LTII have been isolated from humans, cows, buffalo, pigs and ostriches. The LTII enterotoxins share the A1:B5 structure, ADP-ribosyl transferase activity, and immunomodulatory properties of LT1 but differ in their binding specificities.

Putative Virulence Factors of ETEC

The serine protease autotransporter EatA is encoded on the virulence plasmid in human ETEC strains and contributes to virulence in rabbit ileal loops. EatA has over 80% homology with SepA, a virulence-related secreted protein of *Shigella*. Interestingly, a homolog of SepA is encoded on virulence plasmids in certain O149 porcine ETEC (Goswami et al. 2005). Furthermore the plasmids in the porcine ETEC strains encode both drug resistance and the virulence-related factors, STa, Paa, and SepA.

Zhang et al. (2007) found that 60% of *E. coli* from pigs with postweaning diarrhea carried the paa (porcine AE-associated) gene. Paa was initially associated with porcine EPEC and shown to contribute to AE lesion formation but its role in ETEC has not been determined (Batisson et al. 2003).

PATHOGENESIS OF ETEC

ETEC enter the animal by the oral route, and when present in sufficient numbers, colonize the small intestine following attachment by fimbrial adhesins to receptors on the small intestinal epithelium or in the mucus coating the epithelium (fig. 15.4). The ETEC then proliferate rapidly to attain massive numbers to the order of 10^9 per gram of intestine in the mid-jejunum to the ileum. ETEC adhering closely to the intestinal epithelium produce enterotoxins which stimulate the secretion of water and electrolytes into the intestinal lumen. This leads to diarrhea if the excess fluid from the small intestine is not absorbed in the large intestine. ETEC cause severe watery diarrhea, which may lead to dehydration, listlessness, metabolic acidosis, and death.

In some cases, especially in pigs, the infection may progress so rapidly that death occurs before the development of diarrhea, and is referred to as enteric colibacillosis complicated by shock. This phenomenon is probably due to the rapid release of large amounts of LPS by the colonizing ETEC. The lipid A portion of LPS is mostly responsible for the symptoms of shock. LPS stimulates the overproduction of mediators of inflammation including tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6, that cause these symptoms (Whitfield et al. 1994). These mediators promote shock by affecting the vascular endothelium leading to increased vascular permeability. Aggregation and subsequent degranulation of neutrophils activated by these mediators may cause damage to the vascular endothelium. Modulation of the coagulation pathway may lead to fibrin deposition and clot formation.

Enteric ETEC infections may also result in secondary septicemia and manifestations of icterus, petechial hemorrhages in the mucosal membranes, and splenomegaly accompanied by severe diarrhea and dehydration (Fairbrother and Ngeleka 1994). In such cases, ETEC may pass through the intestinal mucosa, probably by endocytic uptake into intestinal epithelial cells or through the intercellular spaces between epithelial cells to locate in the mesenteric lymph nodes before entering the bloodstream, resulting in a generalized infection with bacterial dissemination in extraintestinal organs.

The following sections will highlight specific aspects of the pathogenesis of ETEC infections in various animal species.

ETEC in Pigs

Newborn piglets ingest ETEC found in their environment, especially the mammary glands of the mother and the farrowing crate or pen. These ETEC originate from the feces of piglets with ETEC diarrhea, asymptomatic carrier piglets, or sows (Fairbrother and Gyles 2006). The O serogroups and virotypes of ETEC most commonly associated with diarrhea in newborn piglets are shown in table 15.3. These ETEC belong to two major groups: STa+, F5+, nonhemolytic *E. coli* similar to those that cause diarrhea in neonatal calves, and F4+ ETEC, especially of O group 149, that are also implicated in postweaning diarrhea. Factors that promote development of diarrhea include poor hygiene, inadequate disinfection, a continuous-farrowing system, an ambient temperature of less than 25°C, or excessive air currents. These factors lead to a buildup of pathogenic *E. coli* in the environment or to reduced peristaltic activity and a delay in the passage of bacteria and protective antibodies through the intestine.

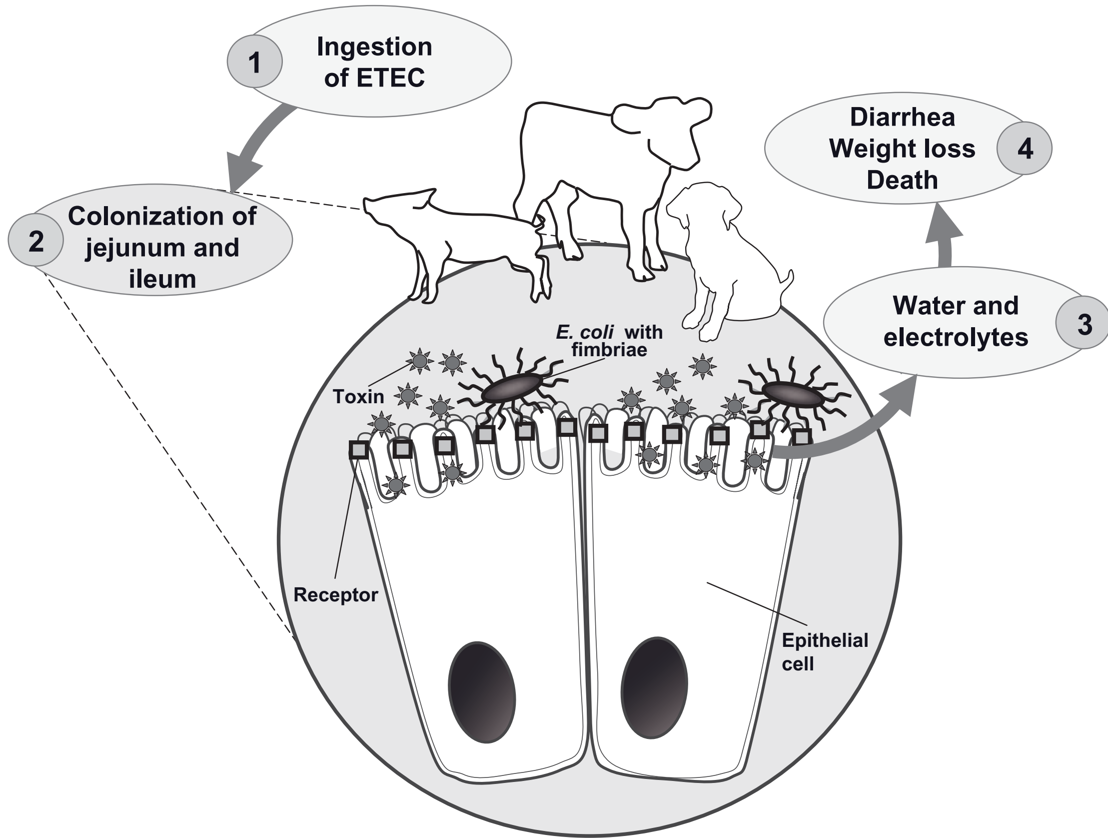

Figure 15.4. Schematic representation of the steps involved in the pathogenesis of ETEC infection. ETEC in the animal's environment are ingested (1), pass through the stomach, adhering to the small intestinal epithelium where they produce enterotoxins (2) that stimulate the secretion of water and electrolytes into the intestinal lumen (3). Loss of water and electrolytes leads to diarrhea, weight loss, and possibly death (4). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal) (See color plate)

In newborn pigs, the pH of the stomach and duodenum is less acidic and the production of digestive enzymes is low compared with older pigs, providing a favorable environment for the rapid multiplication of bacteria such as *E. coli*, including ETEC that may be present in the environment of the piglet. Diarrhea is mostly observed in the first few days after birth and affects one or more animals in a group. A less watery diarrhea may be observed in pigs during the first 1–2 weeks of age, or as early as 2 days post weaning, with low mortality and often with decreased weight gain. In affected piglets of this age group, coinfection with other pathogens such as transmissible gastroenteritis virus, rotavirus, or coccidia, is often observed. F4-producing isolates occasionally proliferate rapidly in the small intestine of young pigs and induce symptoms of shock and rapid death. Enteric colibacillosis complicated by shock occurs in unweaned and recently weaned pigs and manifests as rapid death with cutaneous cyanosis of the extremities, or less acutely with hyperthermia, diarrhea, and anorexia.

Postweaning diarrhea is seen as yellowish or grey fluid and most commonly starts 3–5 days after weaning, lasting up to a week and causing emaciation (Fairbrother et al. 2005). Over several days, most of the pigs in a group may be affected and mortality of up to 25% may be observed in the

absence of antibiotic therapy. In farms where husbandry measures at weaning include addition of higher levels of protein of animal source, plasma, acidifying agents, or zinc oxide, peaks of diarrhea and enteric colibacillosis complicated by shock may be delayed, occurring at 3 weeks after weaning, or even at 6–8 weeks after weaning, at the time when the pigs enter the growing barns (J. M. Fairbrother, unpublished).

Postmortem findings of postweaning diarrhea may be dehydration, dilation of the stomach, gastric infarcts in the mucosa of the stomach, intestinal dilation (by fluid) and congestion, and hyperemia of the small intestine. Intestinal contents vary from yellow to green, watery to mucoid with blood sometimes, and a characteristic odor. The O serogroups and virotypes of ETEC most commonly associated with diarrhea in postweaning pigs are shown in table 15.3. These ETEC are typically hemolytic and are predominantly O149, O8, O141, or O138.

Diet is one of the most important factors influencing the course of the disease in these animals. A diet rich in milk products and energy reduces the duration of the period of lowered feed intake and associated mortality and delays the onset of clinical signs. Dried plasma added to the feed also has a protective effect in reducing the incidence and severity of the diarrhea (van Beers-Schreurs et al. 1992). In contrast, other feed ingredients, such as soybeans, seem to favor the occurrence of postweaning diarrhea. This could be due to the presence of trypsin inhibitors or antigens that induce a localized immune response (Dréau et al. 1994). The latter could result in changes such as a decrease in villus height, deepening of the crypts, and an increase in anti-soya immunoglobulins in the serum, possible predisposing to proliferation of *E. coli*.

The presence of organic acidifiers in the feed can promote a higher mean daily weight gain, feed conversion, and decreased incidence of postweaning diarrhea. The addition of zinc oxide at levels above 2400 ppm in the feed decreases the severity of postweaning diarrhea. ETEC in the environment of the pigs may survive for at least 6 months if they are protected by manure (van Beers-Schreurs et al. 1992), facilitating their spread to other pigs. Infections by other pathogens, such as the porcine reproductive and respiratory syndrome (PRRS) virus may result in immunosuppression, permitting ETEC to cause a septicemia leading to death (Nakamine et al. 1998). Age of weaning may be a predisposing factor for development of postweaning diarrhea, pigs weaned at 2 weeks of age or less being twice as likely to develop diarrhea as those weaned at 5 weeks of age (Svensmark et al. 1989).

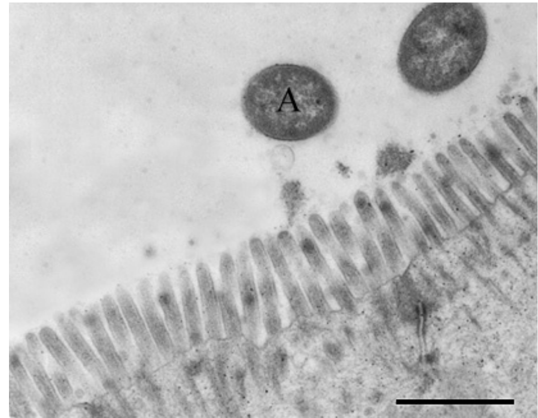

Figure. 15.5. Attachment of ETEC to the ileal epithelial cells of a weaned pig, showing typical less intimate bacterial adherence (A) and no effect on epithelial cells. Bar = 1 µm. (Courtesy of Francis Girard, The *Escherichia coli* Laboratory, Faculté de médecine vétérinaire, Université de Montréal)

On histopathology, layers of *E. coli* are observed adhering to the mucosa of the jejunum and ileum in newborn and postweaning pigs with ETEC infection. Bacteria are usually located at a distance of approximately half to one bacterial width away from the microvilli, as observed on transmission electronmicroscopy (fig. 15.5). Typically, there is no microscopic lesion. ETEC colonize the crypts of Lieberkühn and cover the apex of the villi. In cases of enteric colibacillosis complicated by shock, typical microscopic lesions of hemorrhagic gastroenteritis, congestion, and microvascular fibrinous thrombi and villus necrosis may be observed in the mucosa of the stomach, small intestine, and colon.

ETEC in Cattle and Sheep

In calves and lambs, diarrhea is mostly observed in the first few days after birth. Animals produce large amounts of foul-smelling pasty to watery feces varying from pale yellow to white and occasionally containing flecks of blood. In acute cases, extensive loss of fluid leads to a marked decrease in body weight within 6–8 h of the onset of diarrhea. The intestinal mucosa usually appears normal on histopathology.

The O serogroups and virotypes of ETEC most commonly associated with diarrhea in calves and lambs are shown in table 15.4. These ETEC often produce an acidic polysaccharide type of K antigen, such as K25, K28, K30, or K35), which may enhance intestinal colonization initiated by fimbrial adhesins (Hadad and Gyles 1982). ETEC in calves and lambs typically produce only enterotoxin STa and fimbrial adhesins F5 (K99) and F41 (Nagy and Fekete 1999). These ETEC induce diarrhea in calves only up to a few days of age. Older calves are more resistant, and ETEC infections in calves older than 3 days are usually associated with rotavirus and other viral infections.

As observed in pigs, the reduced gastric acidity in newborn calves increases susceptibility of animals to ETEC infection. Also, gastric pH increases substantially following feeding of calves with milk replacer. Other fimbrial adhesins, such as F17 (Mainil et al. 2000), are less frequently expressed by ETEC isolates from calves with diarrhea and their involvement in attachment of ETEC to enterocytes is less clear. Nevertheless, F17-positive *E. coli* producing the CNF2 can induce diarrhea in newborn, colostrum-deprived calves (Van Bost et al. 2001) and may be involved in the development of diarrhea in calves held in conventional conditions.

ETEC in Dogs

ETEC have been associated with up to 31% of cases of diarrhea in various studies, especially in young dogs, and rarely from healthy control groups of dogs (Beutin 1999). Most of the ETEC isolated from dogs with diarrhea are STa positive, a small proportion of these being also STb positive (Wasteson et al. 1988; Drolet et al. 1994; Hammermueller et al. 1995). No LT-positive ETEC have been reported in association with diarrhea in dogs, a pattern consistent with that seen in neonatal calves. Canine ETEC belong to O serogroups such as O42:H7 that are rarely found among ETEC isolated from other animal species (Wasteson et al. 1988). Few canine ETEC are positive for the ETEC fimbrial adhesins commonly found in other animal species, suggesting that they may carry unidentified species-specific fimbriae.

STEC

Stx is the critical virulence factor in diseases caused by STEC. ED in pigs is the only animal disease for which the role of Stx is clearly established. However, there is evidence to implicate STEC in diarrhea and dysentery in calves and lambs, and cutaneous and renal glomerular vasculopathy (CRGV) in dogs. There have been a few reports of hemolytic uremic syndrome (HUS) in dogs but the link to STEC has not been made. The virulence factors (Stx and the proteins involved in development of the AE lesion) and potential STEC virulence factors are discussed in the following sections.

Virulence Factors

Stx

Stx (also known as verotoxins) are a family of cytotoxic proteins that consist of an approximately 32-kDa A subunit, with N-glycosidase activity, noncovalently associated with a pentamer of B subunits (~7.7 kDa each), which mediate binding to specific receptor molecules (O'Loughlin and Robins-Browne 2001). The two major *E. coli* Stx toxins are Stx1, which is identical to Stx of *Shigella dysenteriae*, and Stx2, which is 56% homologous to Stx1. Variants of the two major types of Stx have been identified based primarily on amino acid composition and biological properties. Differences in animal reservoirs, animal disease, and severity of disease in humans have been related to the various types of Stx. Stx1 has variants Stx1c and Stx1d, with the former carried by sheep and associated with mild or asymptomatic infections in humans. Stx2 has variants Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g. Stx2 and Stx2d (activatable by elastase) are frequently implicated in severe disease in humans; Stx2e is found almost exclusively in strains that cause ED of pigs; and Stx2f is isolated from healthy pigeons.

Combinations of the various Stx (except Stx2e) are found in STEC that are carried by healthy ruminants and some are implicated in diseases in humans. The *stx* genes are carried by temperate lambdoid bacteriophages, along with late genes of the phage genome and are regulated by the phage regulatory circuits (Schmidt 2001; Waldor and Friedman 2005). Induction of phage by agents that affect bacterial DNA or cell wall, including antibiotics, can lead to a massive increase in toxin production (Kimmitt et al. 2000). Stx1, but not Stx2 and its variants, is iron regulated, with enhanced synthesis occurring in low-iron environments. Maximum Stx production *in vitro* occurs at 37°C.

Typically, Stx binds with high affinity to its glycolipid receptor, globotriaosylceramide (Gb3), at the surface of a host epithelial or endothelial cell,

and is internalized by receptor-mediated endocytosis. Following retrograde transfer through the Golgi apparatus, the toxin becomes associated with the rough ER, from which it is released into the cytosol (Sandvig and van Deurs 2002). During transport of Stx, cleavage of the A subunit by the enzyme furin and reduction of a disulfide bond result in separation of a small A2 fragment from the 27-kDa A1 fragment. The A1 fragment interacts with 28S rRNA of 60S ribosomes and catalyzes the removal of a specific adenine residue, thereby inhibiting protein synthesis. Inhibition involves peptide chain termination at the stage of aminoacyl t-RNA binding to the acceptor site on the ribosome. This activity can be lethal for the host cell. A similar effect on bacterial ribosomes (Suh et al. 1998) has led to the speculation that production of Stx may confer a competitive advantage on *E. coli* in the intestine. Stx also induce signaling pathways that result in apoptosis, and activation of nuclear factor κB (NF-κB), Src kinases, and activator protein-1 (AP-1) (Heyderman et al. 2001). Stx also causes enzymatic DNA damage in human endothelial cells by depurination (Brigotti et al. 2007).

The presence of Gb3 on the surface of cells is critical for susceptibility to Stx. Other factors such as the fatty acid composition of Gb3, the internalization of toxin-receptor complexes, and the degradation of internalized toxin also affect cell susceptibility (Lingwood et al. 1998). Stx may bind to uncharacterized protein receptors on the surface of certain epithelial cells, but the role of these proteins in uptake of Stx is unknown (Devenish et al. 1998). Intestinal cell lines such as CaCo2 and T84 transfer Stx from the surface into the cytoplasm without showing signs of cytotoxicity (Philpott et al. 1997). Although the mechanism of transfer is not known, this route is likely to be important in internalization of Stx from the intestinal lumen to the vascular compartment.

There is recent evidence that Stx2 promotes adherence of EHEC O157:H7 to epithelial cells *in vitro* and in the intestine of mice and pigs (Robinson et al. 2006; X. Yin and C. L. Gyles, unpublished). The mechanism appears to be stimulation of production of the intimin receptor nucleolin on the surface of the cells.

The AE Lesion

Certain STEC possess the locus for enterocyte effacement (LEE), a chromosomal PAI that encodes proteins required for a type III secretion system (TTSS), proteins involved in intimate adherence of the bacteria to the host epithelium, and secreted proteins involved in signal transduction in the host epithelial cell (Nataro and Kaper 1998; Dean and Kenny 2009). The LEE_{O157} is a 36.5-kb DNA fragment that consists of 41 genes, organized into five operons (LEE 1–5). A critical component is the *eae* (*E. coli* attaching and effacing) gene that encodes the outer membrane protein intimin (Eae) which functions as an adhesin. Variation in the amino acid sequence in the C-terminus of intimin has lead to recognition of at least 21 variants of intimin in STEC and EPEC. The gene *tir* encodes the translocated intimin receptor (Tir), a protein which is transported through the TTSS into the host cell cytoplasm and reappears on the host cell surface where it acts as a receptor for intimin. Nucleolin and β-1 integrin on host cells are also involved in the interaction of Tir and intimin (Sinclair et al. 2006). This finding provides an explanation for previous observations that intimin from EPEC mediates attachment to the small intestine whereas intimin from STEC mediates attachment to the large intestine.

Interaction between adhesins and host epithelial cells is believed to be an early development in formation of the AE lesion. Long polar fimbriae (LPF; Torres et al. 2002) and Iha (IrgA homologue, Tarr et al. 2000) have been suggested as adhesins that might fill this role. Recently, a type IV fimbria, called hemorrhagic coli pilus (HCP) has been shown to promote adherence of EHEC O157:H7 to bovine and human epithelial cells and to pig and cattle gut explants (Xicohtencatl-Cortes et al. 2007). Antibodies against HCP were detected in the sera of HUS patients but not in the sera of healthy persons.

Following the initial contact, the genes of the LEE are turned on and the TTSS allows secretion of several LEE-encoded and non-LEE-encoded proteins (Nle) into the enterocyte. The LEE-encoded secreted proteins include EspA, EspB, and EspD which form the translocon apparatus, and effectors Tir, EspF, EspG, EspH, EspZ, and Map (mitochondrion-associated protein). So far, 13 Nle effectors have been described. In response to the effector proteins which are injected into or on the surface of the enterocyte, the host cell undergoes extensive cytoskeletal reorganization, involving lysis of the microvilli, formation of a cuplike pedestal, and accumulation of actin and other cytoskeletal proteins beneath the bacteria (Kaper et al.

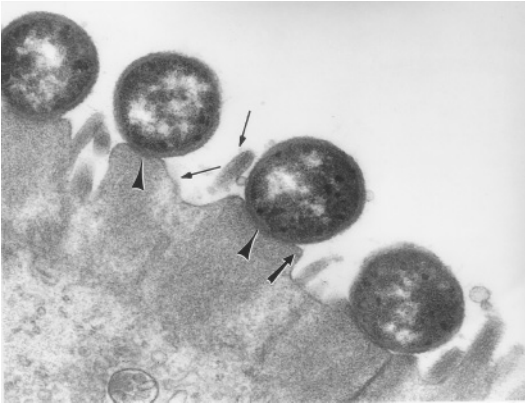

Figure 15.6. Attaching and effacing lesion in the colon of an experimentally infected calf. The intimate attachment of the bacteria, the accumulation of electron-dense material beneath the bacteria, and the effacement of microvilli are evident. (From Sandhu and Gyles [2002], with permission)

1998) (fig. 15.6). Changes in the host cell include increase in intracellular calcium levels, inhibition of absorption of Na^+ and Cl^-, stimulation of Cl^- secretion, and activation of protein kinase C and myosin light chain kinase. Finally, the needle apparatus of the TTSS is lost and intimin and Tir bind to effect intimate contact between bacteria and host cell. Loosening of the tight junctions occurs resulting in greater paracellular permeability. Activation of NF-κB results in synthesis of IL-8 and attraction of polymorphonuclear (PMN) leukocytes which migrate between epithelial cells into the intestinal lumen.

Other Potential STEC Virulence Factors

Hemolysin. Certain STEC, including O157:H7, possess a plasmid-encoded hemolysin, called enterohemolysin or EHEC hemolysin (Ehly), and the STEC implicated in ED in pigs also possess a plasmid that encodes alpha hemolysin. The Ehly operon is positively regulated by GrlA, a LEE-encoded positive regulator of the LEE operon (Saitoh et al. 2008). Antibody to Ehly is produced in humans with EHEC disease and experimental data suggest that the hemolysin may aid survival of STEC in the intestine by making iron available from lysis of erythrocytes.

EAST-1. O157:H7, O26:H11, O111, and O45 EHEC all carry the *astA* gene for the enteroaggregative heat-stable toxin I (EAST-1). This enterotoxin could play a role in the diarrhea caused by these EHEC.

Acid Resistance. O157:H7 and certain other serotypes of STEC are exceptionally acid resistant *in vitro*, being able to survive exposure to pH 2.5 for over 3 h, although there is considerable variation in acid resistance among strains of O157:H7 STEC. At least three acid resistance mechanisms have been identified in O157:H7 STEC—a glutamate-dependent system, an acid-inducible arginine-dependent, and oxidative systems (Audia et al. 2001). In addition, the O polysaccharide contributes to acid resistance (Barua et al. 2002). The acid tolerance of O157:H7 and other STEC is responsible for enhanced survival in acidic foods and resistance to killing by hydrochloric acid in gastric juice, and may be related to the low infectious dose for humans. O157:H7 STEC also carry one or two copies of genes for urease production. These genes are not expressed *in vitro*, but they are functional and may contribute to acid resistance *in vivo*. O157:H7 STEC survive in macrophages, in which they express acid resistance.

Pathogenesis of STEC Disease

General Features

The underlying principles of pathogenesis are the same for a wide range of clinical syndromes observed in animals with STEC diseases (figs. 15.7 and 15.8) STEC in the environment are ingested, pass through the stomach into the intestine, where they colonize and produce Stx. In LEE-positive STEC, formation of the AE lesion is a major feature of intestinal colonization (fig. 15.7). In LEE-negative STEC, binding of STEC to the epithelium occurs in a nonintimate pattern. Varying amounts of the toxin are absorbed into the circulation and cause vascular damage in target organs. Factors that influence production and absorption of toxin from the intestine are largely unknown but induction of Stx phages is likely a significant factor in enhancing toxin production. Other bacterial products, such as LPS, may contribute to pathology by induction of cytokines and upregulation of receptors for Stx. Descriptions of pathogenesis of dysentery in sheep and lambs, CRGV in dogs, and ED in pigs will illustrate variations on this theme.

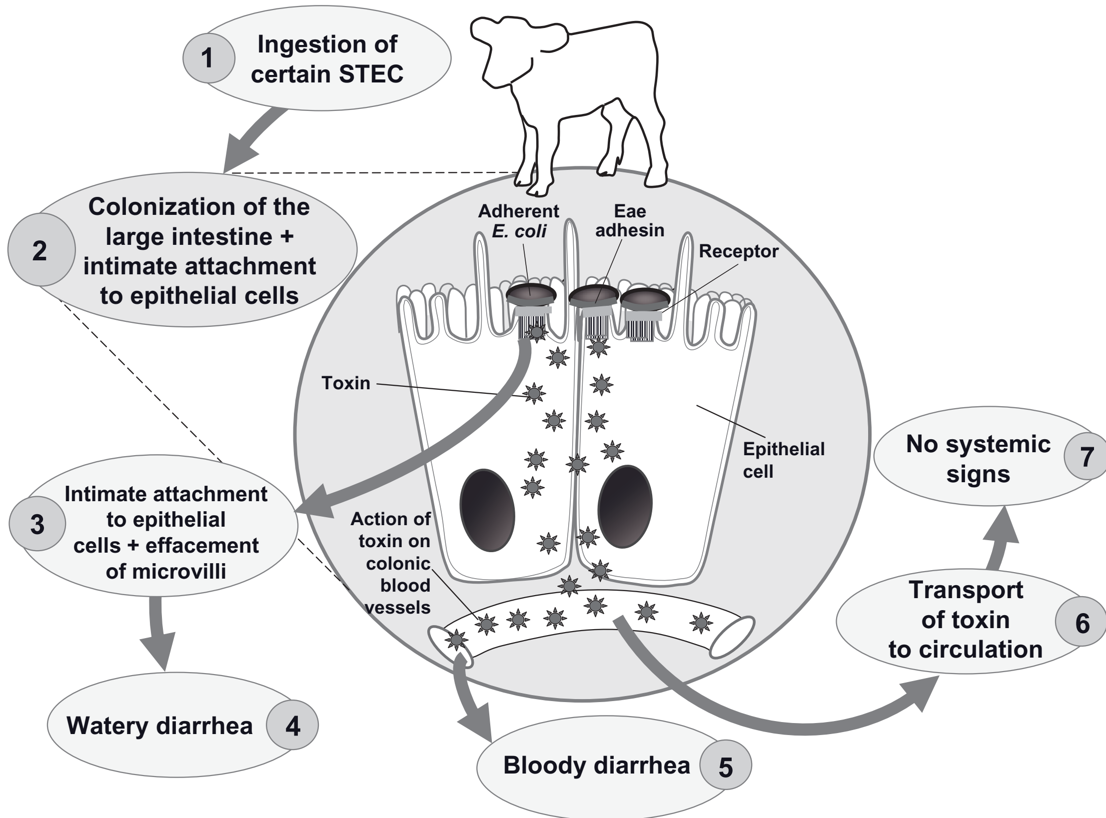

Figure 15.7. Schematic representation of the steps involved in the pathogenesis of STEC infection in dysentery of calves. STEC are ingested, pass through the stomach, and colonize the large intestine through formation of attaching and effacing lesions (1–3). Diarrhea may result from loss of absorptive microvillus surface, activation of secretory activity in epithelial cells, and loosening of tight junctions (4). Shiga toxin 1 and/or 2 is produced in the intestine, and absorbed into the blood; bloody diarrhea is presumed to be due to the action of Shiga toxin locally (5). No systemic signs are observed due to inactivation of the Stx (6). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal) (See color plate)

STEC in Calves and Lambs

A high percentage of cattle and sheep carry STEC without showing any signs of ill health (Naylor et al. 2005; Gyles 2007) (fig. 15.9). This is of enormous public health significance as these animals constitute the major reservoir of STEC that are pathogenic for humans. STEC of serotype O157:H7 are carried at high frequency by cattle but have not been implicated in natural disease, although they may cause disease in calves under experimental conditions (Dean-Nystrom et al. 1997). Some cattle that carry O157 STEC are persistent shedders through colonization of follicle-associated epithelium in a region up to about 5 cm proximally from the junction of the anus and the rectum (Naylor et al. 2003). These cattle shed O157:H7 at levels greater that 10^4 per gram of feces for prolonged periods of time and appear to be major contributors to direct and indirect contamination of food and water.

Dysentery in calves and lambs is clearly attributable to LEE-positive STEC belonging to serotypes O5:NM, O8:H9, O26:H11, O103:H2, O111:NM, O111:H8, and O111:H11 by association with disease and by experimental reproduction of disease (Moxley and Francis 1986; Wray et al. 1989; Dorn et al. 1993). These STEC are typically *stx1* and *eae* positive. Affected calves show AE lesions in the terminal ileum, colon, and rectum, with edema and

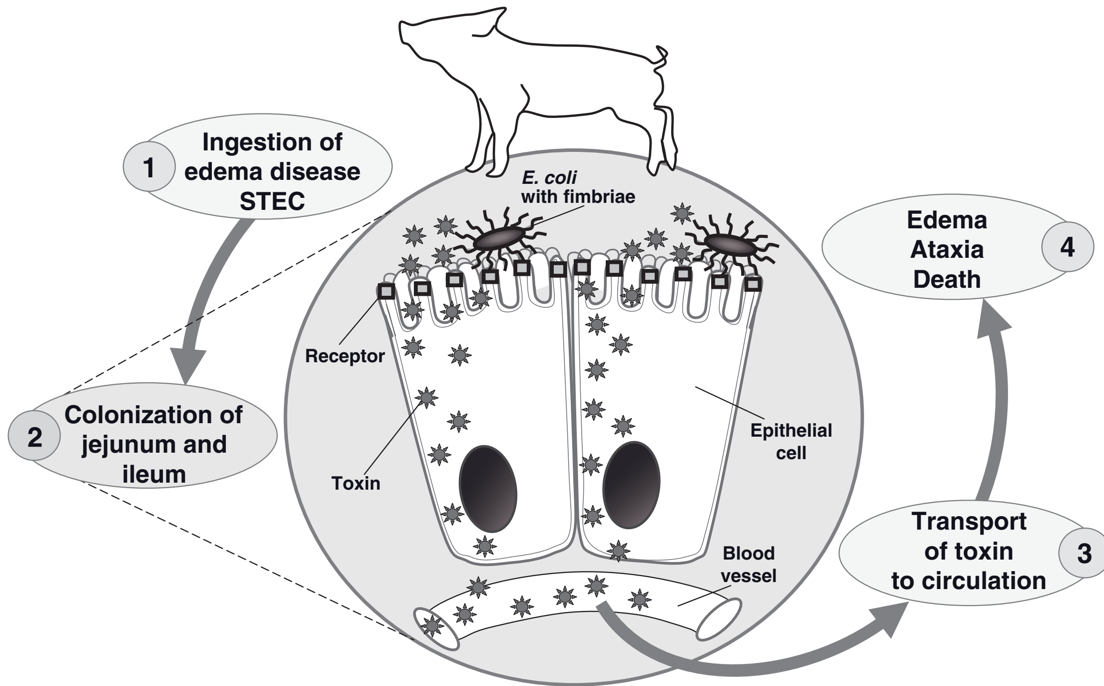

Figure 15.8. Schematic representation of the steps involved in the pathogenesis of STEC infection in edema disease of pigs. The initial steps (1, 2) of ingestion of bacteria and colonization of the intestine are similar to those for ETEC. Shiga toxin 2e (Stx2e) is produced in the intestine and absorbed into the blood (3). The Stx2e binds to receptors on vascular endothelium in the central nervous system and other sites including the stomach and subcutaneous tissues of the forehead and eyelids, giving rise to edema, ataxia, and death (4). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal) (See color plate)

neutrophil infiltration of the lamina propria and an exudate of neutrophils, mucus, and exfoliated epithelial cells in the lumen. The lesions are more severe in colostrum-deprived than in colostrum-fed calves and may be induced with Stx1- or Stx2-positive STEC. Strains of O118:H16 STEC have been epidemiologically associated with diarrhea in calves and have induced disease in inoculated calves.

Calves naturally or experimentally infected with STEC that induce enteric disease show no systemic signs. This is likely due to exclusion of Stx from the ER and trafficking to lysosomes, resulting in inactivation (Hoey et al. 2003).

STEC in Dogs

STEC are present in the feces of healthy and diarrheal dogs (Hammermueller et al. 1995; Beutin 1999) but have not been associated with watery diarrhea in this species. The HUS occurs occasionally in young dogs of several breeds, in which there is a prodrome of bloody diarrhea followed by thrombocytopenia, microangiopathic hemolytic anemia, and anuric acute renal failure (Holloway et al. 1993, Hertzke et al. 1995). HUS occurs in about 5% of the dogs which develop diarrhea. The kidneys of affected dogs show renal proximal tubular necrosis and hemorrhage, and glomerular lesions consisting of hypertrophy, necrosis and loss of capillary endothelial cells, adherence of aggregated platelets to the basement membrane, fibrin thrombi, and fibrinoid necrosis of blood vessels. HUS combined with cutaneous lesions, called CRGV, has been reported in racing greyhounds fed poor-quality ground beef (Carpenter et al. 1988; Cowan et al. 1997; Fenwick and Cowan 1998).

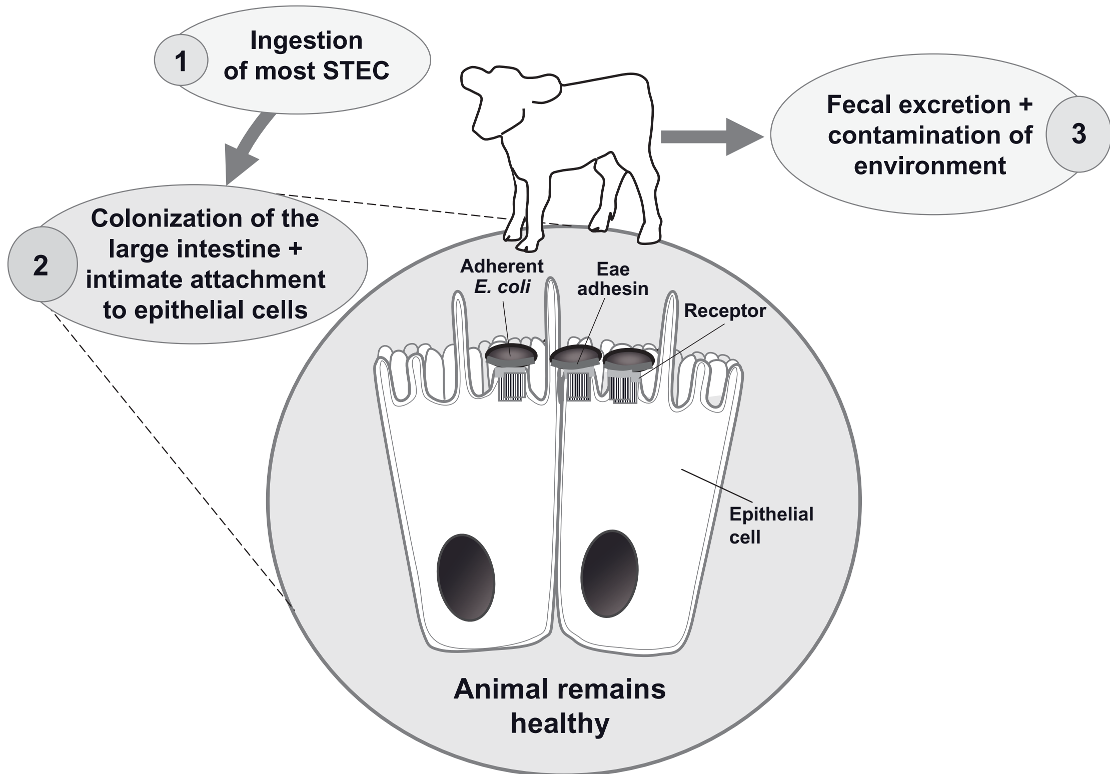

Figure 15.9. Schematic representation of the steps involved in the colonization of the intestine in cattle and sheep and other ruminants by STEC that are pathogenic for humans. These STEC are ingested, pass through the stomach, and colonize the large intestine through formation of attaching and effacing lesions or possibly other adhesins (1, 2). These STEC do not cause disease in natural conditions but may be shed in the feces for long periods and contaminate the environment (3), including food and water consumed by humans. (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal) (See color plate)

O157:H7 and other STEC have been implicated as the cause of this syndrome. The dogs are afebrile and usually present with cutaneous ulcers, edema of the extremities, and bloody diarrhea. The bloody diarrhea has been reproduced by injection of low concentrations of Stx1 or Stx2 (Fenwick and Cowan 1998). Vasculitis of arterioles and necrosis of the epidermis are evident.

STEC in Pigs (ED)

ED is an Stx2e toxemia that results in severe edema in specific sites in pigs that have absorbed Stx2e from the intestine following colonization by an Stx2e-positive *E. coli*. Intravenous injection of pigs with Stx2e at low doses (3 ng/kg body weight) results in the characteristic signs, symptoms, and lesions of ED (MacLeod et al. 1991).

An overview of the association of STEC with ED of pigs is shown in fig. 15.8. ED strains of *E. coli* (EDEC) are ingested from an environment contaminated by sows that carry the bacteria or by pigs that are infected. Colonization develops over 3–6 days and is dependent on nonintimate adherence of the bacteria to epithelial cells on the tips and sides of villi in the small intestine by means of plasmid-encoded F18ab fimbriae (Bertschinger and Gyles 1994). Pigs that lack the intestinal receptors for F18 fimbriae are resistant to ED and can be identified by a PCR assay that detects a specific mutation in the gene for alpha (1,2) fucosyltransferase (FUT1), that may be required for synthesis of a glyconjugate receptor for the F18 fimbriae. Resistance is present in only a low percentage of most breeds of pigs. Age-related expression of receptors for F18

fimbriae (Imberechts et al. 1997b) is consistent with the usual occurrence of ED in older pigs. EDEC usually produce alpha hemolysin *in vitro* and during the course of disease. This cytolysin is not essential for ED (Smith and Linggood 1971) but may contribute to intestinal colonization as *E. coli* that produce alpha hemolysin appear to have a selective advantage compared with nonhemolytic ones in the intestine of recently weaned pigs (Deprez et al. 1986).

Intestinal colonization by EDEC may be aided by host and environmental factors. At the time of weaning the pig's intestinal epithelial cells undergo changes that lead to a period of temporary malabsorption. High-protein diets contribute to an abundance of rich substrate for rapid proliferation of EDEC in the intestine (Smith and Halls 1968; Bertschinger and Gyles 1994). The fastest growing pigs in a litter seem to be most susceptible, and may represent pigs that are consuming most feed and/or are most efficient in absorbing from the intestine. Transportation of pigs and mixing of pigs from a variety of sources also predispose to development of ED, possibly by increasing the stresses on the pigs and the chances of contamination with ED strains of *E. coli*.

Disease is dependent on absorption of Stx2e into the blood stream but there is little understanding of how this occurs. Stx2e does not appear to be absorbed from the intestine under normal conditions, but the addition of deoxycholate to the intestine allows absorption of Stx2e to occur (Waddell and Gyles 1995), and it is possible that bile could influence absorption. Receptors that bind Stx2e are present on enterocytes in the crypts in the small intestine of pigs, but their role in absorption of Stx2e is not known. Stx2e binds preferentially to its specific receptor, globotetraosylceramide (Gb4), in epithelial or vascular endothelial cells, but can also bind to Gb3. In pigs that die of ED, Stx2e can be recovered from the intestinal contents (Sojka 1965), and toxin-mediated damage to vascular endothelium can be identified in target tissues. Strains of EDEC may pass from the intestine to the mesenteric lymph nodes and produce Stx2e toxin there, providing another mechanism for absorption of toxin into the blood.

There are minimal changes to the intestinal epithelium in affected pigs and it is likely that much of the toxin that enters epithelial cells is degraded in the endocytic compartment, with a low percentage of intact toxin passing through the intestinal epithelium. Stx2e binds to red blood cells which may serve to distribute it to various tissues. The absorbed toxin binds to and damages vascular endothelial cells in target tissues, resulting in edema and hemorrhage. The presence or absence of vascular endothelial receptor for Stx2e in blood vessels is a major determinant in the tissue distribution of lesions. However, additional factors determine whether toxin is taken up by a pathway that leads to intoxication (Paton and Paton 1998). Receptors for Stx2e are present in vascular smooth muscle, which undergoes necrosis in target organs in pigs with ED, but it is not known whether the necrosis is a direct result of toxin damage or a secondary effect of vascular endothelial cell damage.

ED may be sporadic or may affect many litters or an entire herd and may be first recognized as sudden death without signs of illness. Some affected pigs become inappetent, develop swelling of the eyelids and forehead, emit a peculiar squeal, and show incoordination and respiratory distress (Sojka 1965; Bertschinger and Gyles 1994). There is no diarrhea or fever.

Pigs that die of ED typically show gross lesions of edema and hemorrhage in some or all of the following sites: the subcutaneous tissues of the eyelids and forehead, the greater curvature of the stomach; the mesenteric lymph nodes, the colonic mesentery, and the brain, especially the cerebellum. Light and electron microscopy identify degenerative changes in vascular endothelial cells, thrombosis in blood vessels, perivascular edema and necrosis of vascular smooth muscle (Bertschinger and Gyles 1994). EDEC recovered from affected pigs most commonly belong to O group 139, and less frequently to O group 138, or 141, although loss of the O antigen does not appear to impair the ability of the organisms to cause disease (Aarestrup et al. 1997). Other O groups which have been associated with ED or possess the *stx2e* genes include O groups 2, 9, 101,107, 120, 121, 125, 147, 149, 154 and 157 (Gannon et al. 1988).

EDEC may or may not be enterotoxigenic. Those strains which are enterotoxigenic function as both ETEC and EDEC (Nagy and Fekete 1999).

EPEC

EPEC are implicated as a cause of diarrhea in several animal species, most importantly, in rabbits, pigs, and dogs. These strains induce AE lesions on

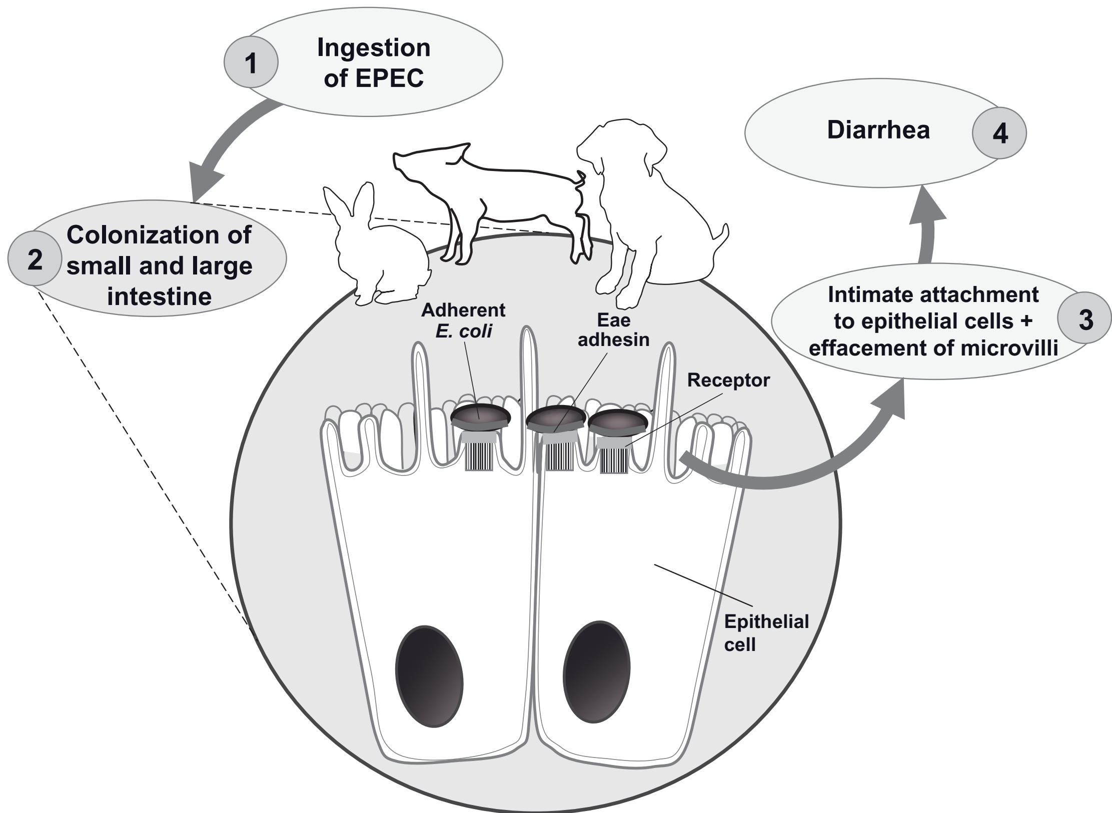

Figure 15.10. Schematic representation of the steps involved in the pathogenesis of EPEC infection in pigs, rabbits, dogs, and other animal species. EPEC are ingested, pass through the stomach, and colonize the small and large intestine through formation of attaching and effacing lesions (1–3). Diarrhea results from loss of absorptive microvillus surface, activation of secretory activity in epithelial cells, and loosening of tight junctions (4). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal) (See color plate)

the intestinal mucosa and are grouped in a category of *E. coli* called attaching and effacing *E. coli* (AEEC), which also includes LEE-positive STEC strains. Healthy cattle, sheep, and pigs appear to be important reservoirs of AEEC, most of which are classified as atypical EPEC (*eae*+ *bfpA*− [bundle-forming pili] and *stx*–) (Krause et al. 2005). Typical EPEC (*eae*+, *bfpA*+) have been isolated from dogs and cats. The roles of various virulence factors and the pathogenic mechanisms of human and animal EPEC have been extensively studied (Nataro and Kaper 1998; Dean and Kenny 2009). A schematic representation of the steps involved in the development of disease due to EPEC in animals is shown in fig. 15.10.

EPEC Virulence Factors

The genes encoding the proteins involved in development of the AE lesion are clustered in the LEE, first described in human EPEC strains (Nataro and Kaper 1998). The LEE is also present in pig EPEC (PEPEC) (Zhu et al. 1994, 1995), rabbit EPEC (REPEC) (Tauschek et al. 2002; Zhu et al. 2001), and dog EPEC (DEPEC) (Goffaux et al. 1999). In the O127:H6 human EPEC strain E2348/69, the LEE is inserted in the *selC* locus at about 82 min on the *E. coli* K12 chromosome, and it is 35-kb long, varying slightly from that of the LEE_{O157}. With some minor differences, the LEE of EPEC mediates AE lesion development as previously described for the

LEE of STEC. EPEC also possess several non-LEE effector genes, as observed for STEC (Dean and Kenny 2009).

The autotransporter EspC enterotoxin located within a PAI at 60 min on the *E. coli* chromosome may be an accessory virulence factor in some EPEC (Mellies et al. 2001). In addition, the *paa* gene, located on the chromosome but outside the LEE, has been found in *eae*-positive PEPEC, DEPEC, REPEC, STEC O157:H7, and to a lesser extent, human EPEC isolates (An et al. 1999; Batisson et al. 2003). Paa, a 27.6-kDa protein located in the periplasm, appears to contribute to the AE process, possibly by influencing the operation of the TTSS (J. Harel, unpublished). In human EPEC, a diffuse adherence fibrillar adhesin, EPEC Afa, appears to function as an initial adhesin that is eventually eliminated from the region of bacteria-host cell contact to allow the typical intimate intimin-Tir interaction and AE lesion formation (Keller et al. 2002). Many human EPEC possess plasmid-encoded type IV bundle forming pili (Bfp) which are responsible for localized adhesion of these bacteria to HeLa cells *in vitro* (Giron et al. 1993) and are proposed to be responsible for initial loose adherence of bacteria to each other and to the target enterocyte apical membrane (Donnenberg et al. 1997). DEPEC strains often also possess a *bfpA*-related gene (Beaudry et al. 1996; Goffaux et al. 2000). Some EPEC (and EHEC) possess a capsule whose presence appears to mask the adherence of EPEC to intestinal epithelial cells (Shifrin et al. 2008). Interestingly, Ler which positively regulates the genes of the LEE downregulates capsule synthesis. It is suggested that initially there is partial shielding which allows injection of proteins through the TTSS, and subsequently there is reduction of the capsule allowing intimin to bind to its receptor.

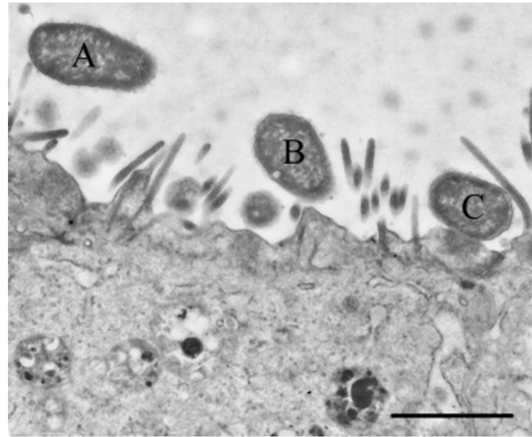

Figure 15.11. Attachment and effacement of PEPEC to the ileal epithelial cells of a weaned pig, showing initial loose bacterial attachment (A), subsequent pedestal formation (B), and more intimate bacterial adherence with electron dense actin polymerization beneath the bacterium and microvillus effacement (C). Bar = 2 μm. (Courtesy of Francis Girard, The *Escherichia coli* Laboratory, Faculté de médecine vétérinaire, Université de Montréal)

EPEC Pathogenesis

EPEC attach loosely to the intestinal epithelial cells (fig. 15.11, bacterial cell A), probably by means of specific adhesins such as AF/R1, AF/R2, and Ral in the rabbit, and Bfp in the dog. The bacterial adhesins involved in this step have not been well characterized in EPEC from animal species other than the rabbit. A signal is then delivered from the bacteria to the epithelial cells, probably via the TTSS and secreted proteins (Viswanathan et al. 2009). The signal results in increased intracellular levels of calcium, phosphorylation of certain epithelial cell proteins and activation of kinases and of the Tir receptor binding activity. The outcome is an intimate bacterial attachment to the epithelial cells due to recognition of Tir and host cell receptors by the bacterial intimin, and cytoskeletal changes such as accumulation of polymerized actin directly beneath the adherent bacteria (fig. 15.11, bacterial cells B and C). There is a subsequent effacement of microvilli in the proximity of the bacterial attachment and bacteria are often observed to sit on pedestal-like structures which can extend from the epithelial cell in a pseudopod-like structure. The signaling activity also results in an influx of PMN neutrophils at the site of bacterial adherence.

The mechanisms by which EPEC induce diarrhea are not well understood. The loss of absorptive microvilli in the AE lesion could lead to diarrhea due to malabsorption (Nataro and Kaper 1998). However, the rapid onset of diarrhea suggests that a more active secretory mechanism is also involved and may result from the effect of EPEC signaling activity on intracellular mediators of intestinal ion transport, such as calcium, inositol phosphates, and tyrosine kinase. The development of diarrhea may also be due, in part, to an increased

permeability of tight junctions between epithelial cells, a localized inflammatory response at the lesion site, or chloride secretion following PMN leukocyte transmigration.

EPEC in Pigs

An overview of the interaction of PEPEC with the pig is shown in fig. 15.9. PEPEC are associated with postweaning diarrhea in pigs (Zhu et al. 1994, 1995), and in Canada, they often belong to serogroup O45 or O103. In Hungary, Malik et al. (2006) found no difference in the frequency of occurrence of *eae*+ *E. coli* in diarrheic and non-diarrheic pigs, but reported that serotype O123:H11 constituted 40% of the intestinal isolates. PEPEC attach to and efface the microvilli of the small and large intestinal mucosa in postweaning pigs (Helie et al. 1991). Several predisposing factors, such as a weaner diet containing soybean and field peas or PRRS virus infection, may enhance bacterial colonization and development of AE lesions. Host immune status is an important factor in the development of AE lesions. Thus, colostrum-deprived piglets are more susceptible to infection than those having received colostrum and dexamethasone immunosuppression is required for development of AE lesions in a postweaning *in vivo* PEPEC pig infection model (Girard et al. 2005).The mechanisms by which PEPEC cause diarrhea and the role of co-infection with other pathogens are not known. However, the similarity to human EPEC both in terms of the pattern of intestinal colonization, the type of lesion produced and the presence of Eae on bacteria strongly suggest that porcine and human EPEC share similar pathogenic mechanisms.

For PEPEC infections, histopathological lesions range from mild and scattered through the large and small intestine, to severe and involving mostly the cecum and colon. They include light to moderate inflammation of the lamina propria, enterocyte desquamation and some mild ulceration, and light to moderate villus atrophy in the small intestine. Extensive multifocal bacterial colonization of the surface epithelium by a thin layer of coccobacilli, often oriented in a palisade pattern, is observed. Typical intimate attachment of bacteria to intestinal epithelial cells and effacement of microvilli are observed on electron microscopy (fig. 15.11).

EPEC in Dogs

DEPEC are the pathogenic *E. coli* most commonly associated with diarrhea in dogs (Janke et al. 1989; Drolet et al. 1994; Beaudry et al. 1996; Wada et al. 1996; Beutin 1999; Goffaux et al. 2000). There is at least one report of identical EPEC strains having been isolated from a diarrheic puppy and and a young child in the same household. EPEC have also been isolated from cats (Goffaux et al. 2000). The DEPEC strains possess the LEE and often also possess a *bfpA*-related gene. Cases have a history of gastrointestinal disease associated with histological and bacteriologic evidence of AEEC (Janke et al. 1989; Drolet et al. 1994; Wada et al. 1996). Typical AE lesions are observed in the jejunum and ileum, to a lesser extent in the large intestine, and have been reported in the stomach of affected dogs. Many of these dogs originate from kennels and pet shops and are aged between 1.5 and 3 months. Coinfection with other enteric pathogens such as canine distemper virus, canine parvovirus, or coccidia is often observed (Drolet et al. 1994; Wada et al. 1996). DEPEC isolates belong to heterogeneous serogroups, including O45, O49:H10, O115, O118:NM, and O119 (Beutin 1999). Hence, DEPEC strains, although often possessing similar virulence determinants to those of human EPEC, belong to different serotypes.

EPEC in Rabbits

REPEC are the only important class of pathogenic *E. coli* in rabbits, being one of the principal infectious agents in diarrheic rabbits and causing 25–40% losses. Certain serotypes of REPEC, such as O109:H2 and O8:H?, are mainly pathogenic for suckling rabbits whereas other serotypes, such as O26:H11, O20:H7, O109:H7, O153:H7, O128:H2, and O132:H2, are associated predominantly with disease in weaned rabbits. REPEC of serotypes O2:H6, O15:H–, O103:H2 are associated with disease in both suckling and weaned rabbits (Peeters 1994; Blanco et al. 1997; Milon et al. 1999). In general, O109:H2 strains tend to cause severe and lethal diarrhea in suckling but not weaned rabbits, whereas O15:H–, O103:H2, and some O26:H11 strains tend to induce severe diarrhea with a high mortality rate in weaned rabbits (Milon et al. 1999). REPEC strains of other serotpyes tend to cause a mild diarrhea with possible weight loss.

Adhesins that have been associated with REPEC include AF/R1 (adhesive/rabbit 1), AF/R2 (adhe-

sive factor/rabbit 2), Ral (REPEC adherence locus) (Dow et al. 2005), and LPF. Both Ral (Krejany et al. 2000) and LPF produced by an O15:H– REPEC played a role in development of diarrhea in rabbits (Newton et al. 2004). Inactivation of the genes that encode these fimbriae reduced early colonization and severity of diarrhea in experimentally infected rabbits but permitted development of typical AE lesions.

Any factor which increases cecal pH or decreases cecal volatile fatty acid levels favors REPEC infection (Peeters 1994). Such factors include feed composition, stress, cold, and lack of drinking water. Coinfections such as rotavirus infection or coccidiosis result in increased numbers of *E. coli* in the cecum and favor the development of diarrhea due to REPEC in rabbits. In suckling rabbits, diarrhea due to REPEC occurs at 3–12 days of age, with mortality of up to 100% within a litter. In weanling rabbits, diarrhea is observed at 5–14 days after weaning, with mortality of 5–50% and decreased weight gain in surviving animals.

In suckling rabbits, typical AE lesions are observed over the entire length of the small and large intestines. In weanling rabbits, typical AE lesions are found mostly in the cecum, and to a lesser extent in the colon and ileum. RPEC strains possess *eae* and other genes of the LEE (Blanco et al. 1997; Milon et al. 1999) The full LEE locus has been demonstrated in several O15:H– and O103:H2 strains (Zhu et al. 2001; Tauschek et al. 2002). REPEC strains also often possess one of the fimbrial adhesins AF/R1 (Pohl et al. 1993b; Robins-Browne et al. 1994; Von Moll and Cantey 1997), AF/R2 (Pillien et al. 1996; Fiederling et al. 1997), or Ral (Krejany et al. 2000), which appear to be involved in bacterial adherence to the rabbit intestinal epithelium. These fimbriae may be involved in initial attachment of REPEC to the enterocyte apical membrane, as proposed for BfpA of human EPEC (Milon et al. 1999).

EXPEC

This category includes *E. coli* implicated in a wide range of infections including septicemia, and infections of the urinary tract, the genital tract, and the mammary gland. ExPEC recovered from disease in humans and animals share several virulence factors and animals may be reservoirs of some of these pathogens that cause disease in humans. ExPEC are found in the normal intestinal microflora. In contrast to ETEC, EPEC, and STEC, they are not characterized by the presence of a particular virulence factor or group of factors but usually possess a large number of virulence factors which may vary greatly between strains. These include factors contributing to bacterial colonization, invasion, iron acquisition, resistance to the bactericidal effects of complement and phagocytosis, and toxic activity. A schematic representation of the steps involved in the development of disease due to ExPEC in animals is shown in fig. 15.12.

Septicemic Disease

Septicemic *E. coli* (SEPEC) belong to a limited number of serotypes (Orskov and Orskov 1992). In certain circumstances, such as in young animals which have received inadequate colostral antibodies, these strains colonize the intestinal mucosa and traverse the intestinal epithelial barrier by pinocytosis or transepithelial migration to mesenteric lymph nodes (Gay and Besser 1994). Bacteria persist and multiply in the blood and other extraintestinal sites, partly because of their ability to adapt to and grow in the iron-restricted extracellular environments of the host, largely due to the presence of the aerobactin iron acquisition system (Griffiths 1994). Persistence and multiplication of SEPEC is also promoted by their ability to resist the bactericidal effects of complement and phagocytosis.

One of the most important determinants of serum resistance of SEPEC is the chemical structure and length of the O polysaccharide, C3b, and C5b-C9 being bound by long O polysaccharide chains, thus preventing insertion of the membrane attack complex into the bacterial cell membrane (Whitfield et al. 1994). Nevertheless, the presence of smooth LPS is not sufficient for serum resistance in all O serogroups (Stawski et al. 1990). Certain capsular antigens, such as the K1 polysaccharide, also contribute to serum resistance. Other virulence factors, such as the ColV plasmid-encoded outer membrane proteins TraT and Iss, are also associated with complement resistance.

The most important determinant of resistance to phagocytosis of SEPEC is the capsule due to its hydrophobicity and negative charge (Horwitz and Silverstein 1980). Other factors, such as P fimbriae (Tewari et al. 1994) and certain O antigens (Burns and Hull 1999), also contribute to the ability of SEPEC to avoid phagocytosis. Type 1 fimbriae promote bacterial adhesion to phagocytic cells by

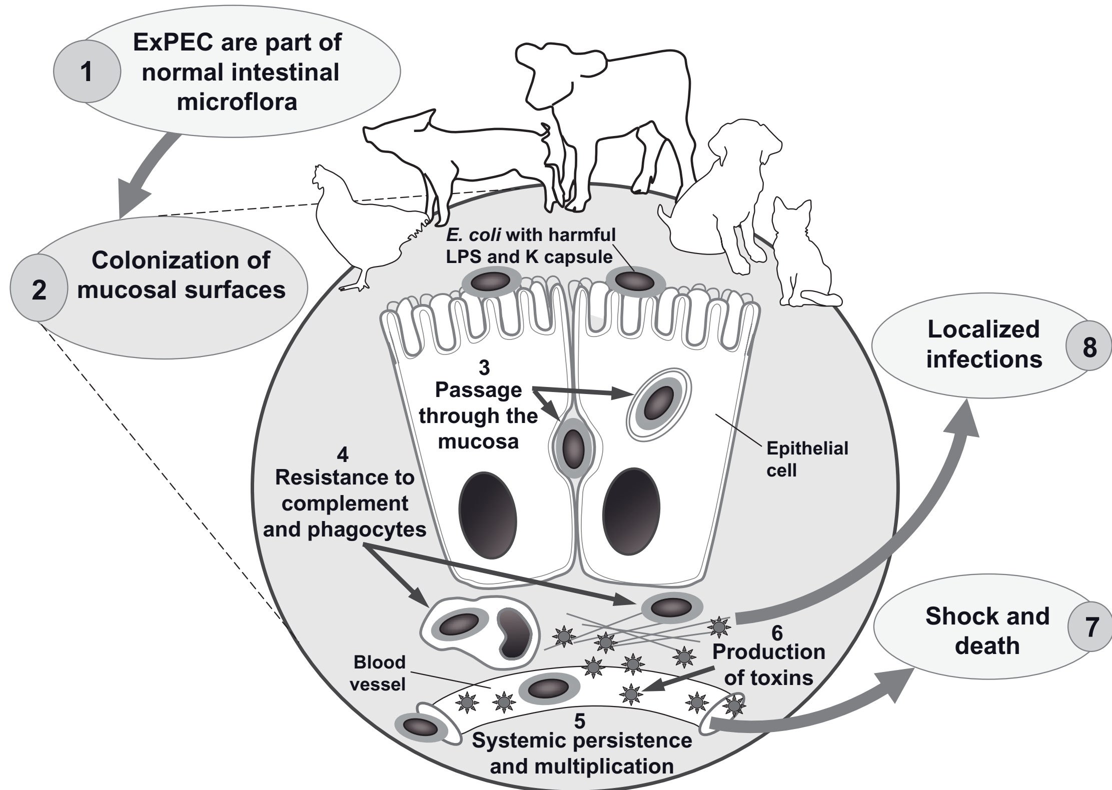

Figure 15.12. Schematic representation of the steps involved in the pathogenesis of ExPEC infection. Ingested bacteria colonize the intestine (1, 2) then pass through and between epithelial cells to gain access to the underlying tissue (3). Resistance to complement and phagocytes permits survival of the bacteria (4) which are transported to distant organs via the blood stream (5). Production of toxins, notably LPS, locally and in the blood stream lead to localized and generalized disease (7, 8). (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal) (See color plate)

lectin–carbohydrate interactions, although the role of these fimbriae in resistance to phagocytosis is still controversial. As described for ETEC, LPS is responsible for the symptoms of shock associated with septicemia.

SEPEC from calves and pigs often produce the fimbrial adhesins F17 (Lintermans et al. 1988), P, S, F1C, or F165 (Harel et al. 1995; Dozois et al. 1997), or other adhesins such as the surface antigen CS31A (Girardeau et al. 1988; Korth et al. 1991), the afimbrial AfaE-VIII adhesin (Girardeau et al. 2003), or curli (Olsèn et al. 1993) (tables 15.3 and 4). Certain SEPEC are hemolytic and often produce colicin V, CNF1, or CNF2 (Fairbrother and Ngeleka 1994; De Rycke et al. 1999), and cytolethal distending toxin (CDT) and may possess the high-pathogenicity island (HPI) present in pathogenic *Yersinia* (Dezfulian et al. 2003; Girardeau et al. 2003).

SEPEC in Pigs

E. coli septicemia occurs in neonatal pigs and less frequently in suckling pigs (Fairbrother and Ngeleka 1994). It is characterized by an acute generalized infection, sometimes with diarrhea at the terminal stage, with signs of shock, often followed by death in 3–8 h, with fatality of up to 100%. The infection may become localized causing polyarthritis, pneumonia, metritis, abortion (Pohl et al. 1993a; Bilkei et al. 1994), or meningitis.

In acute primary septicemia, there may be no gross lesions but congestion of the intestine, the mesenteric lymph nodes, and the extraintestinal

organs may be observed. In subacute cases, subserous or submucosal hemorrhages and fibrinous polyserositis with gross lesions of pneumonia are usually observed, and may be accompanied by fibrinopurulent arthritis and meningitis (Fairbrother and Ngeleka 1994).

In pigs manifesting symptoms of shock and rapid death, typical histopathological lesions of septicemia such as hemorrhagic gastroenteritis, congestion, renal hemorrhage, and thrombi in the mucosa of the stomach and small intestine are observed. These lesions probably result from the rapid release of bacterial LPS from the intestine into the circulation.

The predominant serotypes and virotypes associated with septicemia in pigs are shown in table 15.3. The roles of some of the virulence determinants are only partially understood. O antigen capsule and fimbrial antigen in $F165_1$ are associated with bacterial survival in the extraintestinal organs and in the bloodstream of infected piglets and with pathogenicity for piglets (Ngeleka et al. 1992, 1993). $F165_1$ promotes bacterial adherence to porcine PMN leukocytes *in vitro* but enhances resistance to phagocytosis (Ngeleka et al. 1994). One mechanism for this resistance appears to be an inhibition of the oxidative response (Ngeleka and Fairbrother 1999). O antigen capsule K"V165" confers resistance to serum and to phagocytosis by PMN leukocytes (Ngeleka et al. 1992, 1994).

SEPEC in Calves

E. coli septicemia occurs in calves in the first few days of life and in lambs at 2–3 weeks of age (Gay and Besser 1994; Fecteau et al. 2009). Bacteria enter the host across the intestinal epithelium, or through the umbilicus. The clinical signs and localizations are as described for pigs. Bacteria are excreted in nasal secretions, urine, and feces in affected animals. On postmortem of calves, in peracute cases there are usually petechial hemorrhages on the epicardium and serosal surfaces and there may be pulmonary edema and hemorrhage and enlargement of the spleen. Fibrinous polyarthritis and meningitis may be observed in chronic cases.

The predominant serogroups and virotypes associated with septicemia in calves are shown in table 15.4. Bovine SEPEC often carry the P, F17, AfaE-VIII, or CS31A adhesins which may promote mucosal adherence and allow these bacteria to compete at the mucosal surface favoring host invasion and entry into the blood (Gay and Besser 1994). These adhesins are frequently present on *E. coli* isolates from the blood of bacteremic calves, although their presence is not always associated with calf mortality (Fecteau et al. 2001). Hence, these factors could contribute to the ability of SEPEC to translocate from the intestine and survive in the blood in certain circumstances, but may not be sufficient to induce septicemia and death.

SEPEC in Chickens

Respiratory tract infection with avian pathogenic *E. coli* (APEC) results in depression and fever in birds of 4–9 weeks of age and may result in extensive economic losses with up to 20% mortality (Dho-Moulin and Fairbrother 1999). Air sacs of infected birds are thickened and often have a caseous exudate on the respiratory surface. On histopathology, edema is the earliest change and initial infections are characterized by airsacculitis with a serous to fibrinous exudate, an initial infiltration with heterophils and a subsequent predominance of macrophages, which is frequently followed by a general infection commonly resulting in perihepatitis and/or pericarditis. APEC may infect the oviduct of laying birds via the left abdominal airsacs leading to salpingitis and loss of egg-laying ability. APEC may sporadically invade the peritoneal cavity via the oviduct, leading to peritonitis and death in caged layer hens. APEC may also cause a syndrome called the swollen head syndrome characterized by gelatinous edema of the facial skin and periorbital tissues, and caseous exudate in the conjunctival sac, facial subcutaneous tissues, and lacrimal gland.

APEC respiratory tract infection occurs via inhalation of feces-contaminated dust. It is secondary to infection with one or more of the respiratory tract agents Newcastle disease virus, infectious bronchitis virus, and *Mycoplasma gallisepticum*. Susceptibility of birds to APEC infection is increased by deciliation of the epithelial cells of the upper respiratory tract following exposure to ammonia and dust in the environment of the birds. Dominant O serogroups among APEC are O1, O2, and O78, but there is a wide range of serogroups and many strains are nontypable.

APEC adhere to epithelium of the respiratory tract by means of fimbriae (Dho and Lafont 1984; Dozois et al. 1994) and enter the bloodstream via the lungs and airsacs (Pourbakhsh et al. 1997a) to reach internal organs. Type 1 fimbriae are mostly

expressed by bacteria colonizing the trachea, lungs, and air sacs, but not those colonizing deeper tissues or blood (Pourbakhsh et al. 1997b). However, intramuscular or intranasal vaccination with the type 1 FimH adhesin failed to protect chickens from challenge with a type 1 fimbriae-positive APEC, despite inducing a strong systemic immune repsonse (Vandemaele et al. 2005). On the other hand, P fimbriae of serotype F11, produced by some APEC strains, are expressed by bacteria colonizing the air sacs, lungs, and internal organs, but are not expressed by those colonizing the trachea, suggesting an involvement in bacterial attachment in deeper tissues. The *pap* operon of an O1:K1 APEC was part of a PAI which included genes *tia* and *ireA*, and was immediately downstream of the *kps* gene cluster that encodes the biosynthesis of the K1 polysialic acid capsule (Kariyawasam et al. 2006).

A newly recognized fimbria, called Stg, appears to be involved in colonization of air sacs (Lymberopoulos et al. 2005). Curli promote binding to the major histocompatibility complex class I (MHC-I), extracellular matrix and serum proteins, and avian intestinal cells (La Ragione et al. 2000), suggesting that they may contribute to APEC infection. Temperature-sensitive hemagglutinin (Tsh), a serine protease autotransporter virulence-associated protein (Dozois et al. 2000; Ngeleka et al. 2002), is associated with APEC and is frequently encoded by a gene on ColV-related virulence plasmids. Chicken infection studies using a wild-type strain and its isogenic *tsh* knockout mutant suggest a possible role for Tsh in the early stages of respiratory infection.

Resistance to the bactericidal effects of complement has been associated with APEC isolates, particularly those originating from septicemic birds (Dho-Moulin and Fairbrother 1999). Pourbakhsh et al. (1997a) showed that K1-positive APEC strains were more resistant to the bactericidal effect of serum than APEC strains expressing other K antigens. The K1 antigen is frequently associated with APEC of serogroups O1 and O2. Studies with isogenic mutants have confirmed the role of K1 capsule, as well as that of the O78 LPS, in resistance of APEC to the effects of serum (Mellata et al. 2003a). The *iss* gene occurs much more frequently in isolates from birds with colibacillosis than in faecal isolates from healthy birds (Nolan et al. 2003). However, this gene might not play a major role in resistance to serum (Mellata et al. 2003a), but may be a marker for a larger pathogenicity unit (Nolan et al. 2003).

Avian air sacs have no resident cellular defense mechanisms and must rely on an inflammatory influx of heterophils as the first line of cellular defense, followed by macrophages (Toth and Siegel 1986). *In vivo* experiments showed that APEC were present in macrophages, but occasionally were also free in the air sac lumen and interstitium of infected chickens. In the airways, bacteria were free within the lumen and mixed with heterophils, erythrocytes, and fibrin (Pourbakhsh et al. 1997a). Results of several studies suggest that resistance to phagocytosis may be an important mechanism in the development of avian septicemia (Dho-Moulin and Fairbrother 1999). Type 1 fimbriae play an important role in the promotion of initial phagocytosis, but also in the protection of bacteria from subsequent killing, at least in heterophils (Mellata et al. 2003b). K1 capsule, O78 antigen, and P fimbriae participate in initial avoidance of phagocytosis. In addition, O78 antigen and P fimbriae contribute to subsequent protection against the bactericidal effects of phagocytes after bacterial association has occurred.

Iron acquisition is important in the virulence of APEC. This is illustrated by the presence of genes for four iron acquisition systems (aerobactin, Sit, salmochelin, and temperature sensitive hemagglutinin) in the virulence plasmid of the O78:K80 strain APEC-1 (Mellata et al. 2009). In addition, genes for ferric yersiniabactin uptake and iron-repressible protein are very frequently present in APEC. The current state of knowledge of APEC virulence factors has been recently reviewed (Dziva and Stevens 2008).

Avian Cellulitis

APEC are also associated with asymptomatic cellulitis of the lower abdomen and thighs. Gross lesions are typically 3–6 cm in diameter, in the skin of the postventral region, and tend to be unilateral with moderate to marked thickening of the skin (Messier et al. 1993). The skin is discolored and yellowish fibrinocaseous plaques are found in the subcutaneous tissues underlying the skin lesions.

On histopathology, moderate hyperkeratosis and hyperplasia of the epidermis, marked fibrous thickening of the dermis with evidence of neovascularization, and diffuse infiltration of mononuclear cells and heterophils are observed in the lesions. There may be focal ulceration of the epidermis and coalescing granulomas, characterized by the

accumulation of a fibrinocaseous exudate surrounded by a thin layer of epithelioid and multinucleated giant cells, in the subcutaneous tissues. The exudate in the subcutaneous tissues consists of cellular debris, fibrous tissue, inflammatory cells, and short gram-negative rods. Feather follicles may be involved.

Cellulitis does not seem to affect the growth of the bird but results in complete or partial condemnation of the carcass at processing (Messier et al. 1993). The lesion is initiated by a break in the integument, in some cases due to a scratch from another bird, followed by bacterial contamination. Bacterial adherence to the deeper and superficial tissue layers of the skin appears to be important in the development of lesions and may be promoted by type 1 fimbriae. Cellulitis has been reported to be characterized by seasonal variation, and some climatic conditions may be predisposing factors (Gomis et al. 2000). Broiler chickens appear to be more susceptible to cellulitis than are leghorn chickens (Olkowski et al. 2005). The susceptibility of broilers was associated with structural weakness of the skin and a less effective innate immune response.

Urinary Tract Infections

E. coli is the pathogen that is most frequently implicated in urinary tract infections (UTIs) in dogs, cats, and human beings. Infection, which is more common in dogs than in cats, is most frequently manifested as cystitis but urethritis, pyelonephritis, and prostatitis are also seen. There are many studies on virulence factors of canine UPEC (Low et al. 1988; Yuri et al. 1998, 2000; Johnson et al. 2001b, 2001c, 2008), but very few on pathogenesis (Yuri et al. 2000). However, remarkable similarities between the virulence-associated genes in canine and human UPEC suggest that major aspects of pathogenesis are similar between UPEC of these host species. Indeed, several studies support the conclusion that dogs may be a source of UPEC that cause UTI in humans (Low et al. 1988; Johnson et al. 2001a, 2001b, 2001c, 2008; Kurazono et al. 2003; Johnson and Clabots 2006).

Cystitis due to UPEC results from invasion of the bladder by bacteria present in the rectum, multiplication of the bacteria in the bladder, the inflammatory response to the UPEC, and tissue damage inflicted by the UPEC (fig. 15.13). Much of our understanding of the disease process derives from experimental infections involving human or canine UPEC strains introduced into the bladder of mice, referred to as a mouse model of ascending UTI.

The major steps in pathogenesis involve adherence of UPEC to the bladder epithelium, colonization, avoidance of host defense, and damage to the host tissues (Smith et al. 2007). Urovirulent *E. coli* from the feces initially colonize the periurethral area then move up into the urethra. The normal flora in these sites, including lactobacilli, may compete with the UPEC for colonization. Continued migration takes the UPEC into the bladder, where they adhere to bladder epithelial cells. Not surprisingly, motility is a factor in the virulence of UPEC. Cytokines, including IL-8, are produced by the host and attract a strong neutrophil response. UPEC employ systems that protect against oxidant and osmotic stress and P fimbriae and the K1 and K5 capsules impart some resistance to phagocytosis. Nevertheless, some UPEC are phagocytosed and killed by neutrophils and some ExPEC are able to survive in neutrophils. Invasion of epithelial cells provides significant protection and longer term survival. Colonization requires recovery of iron from the host, and this is done through a variety of siderophores. Damage to bladder epithelium is largely the result of the inflammatory response including the toxic products of the neutrophils, but toxins produced by UPEC contribute as well. Infection does not often ascend to the kidneys.

E. coli that cause UTI typically originate from the dog's own intestinal tract and are characterized by possession of a cluster of virulence-related genes (Feria et al. 2001; Johnson et al. 2003; Kurazono et al. 2003). Human UPEC strains possess a genome that is 6–13% larger than the *E. coli* K12 genome and many virulence-related genes are encoded in PAIs. PAI-associated genes implicated in virulence encode P fimbriae, S fimbriae, hemolysins, CNF1, and iron-sequestering systems. The genes for alpha-hemolysin and aerobactin may be found on either PAIs or plasmids. Virulence-related factors that are encoded on the core genome include type 1 fimbriae, specific O antigens (commonly O1, O2, O4, O6, and O25), and enterobactin. Among the canine UPEC that possess P fimbriae, the *papG* allele III tip adhesin is by far the most frequent. Various UPEC may possess different combinations of virulence factors. Studies with human UPEC have identified PAI I with the *hly* (alpha-hemolysin) gene alone or in combination with *pap* (pyelonephritis-associated pili), or genes for other adhesins; PAI II

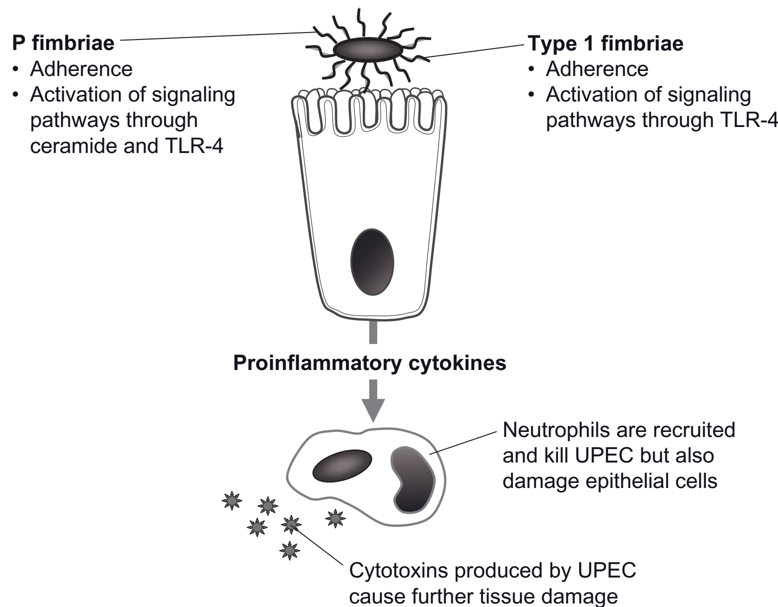

Figure 15.13. Uropathogenic *E. coli* (UPEC) in cystitis. UPEC may adhere through binding of the FimH adhesin of type 1 fimbriae to mannosylated glycoproteins and to TLR-4 (coreceptor) on the surface of uroepithelial cells and activate TLR-4. P fimbriae bind to Galα(1–4)Galβ receptors in ceramide-anchored glycosphingolipids, resulting in the release of ceramide which activates TLR-4. Activation of the epithelial cells results in secretion of cytokines, notably IL-6 and IL-8, which recruit neutrophils to the area. The neutrophils engulf and kill the UPEC. (Courtesy of Jacinthe Lachance, Reference Laboratory for *Escherichia coli*, Faculté de médecine vétérinaire, Université de Montréal.) (See color plate)

with *hly*, *prs* (*pap*-related sequence), *cnfI*, or *hly*, *prf*, or *pap*, *bfrB* (bacterioferritin); PAI III with *iro* (salmochelin), *sfa* (S fimbriae); PAI IV with *ybt* (yersiniabactin); PAI V with *kps* (capsule); and HPI with *ybt* (Oelschlaeger et al. 2002).

The virulence-related factors play important roles as adhesins that mediate adherence to urinary tract epithelium, iron-scavenging systems that enhance survival in low-iron environments, and cytotoxic proteins that damage tissues. Pathogenesis involves a timely and stepwise expression of these virulence factors in hosts with formidable defense mechanisms which include the mechanical barrier imposed by the epithelium, flushing by frequent voiding of urine, and the inflammatory response.

Adherence to the mucosa of the urinary tract is an early step in pathogenesis and is often mediated by type 1 fimbriae, which are present on most *E. coli*, and therefore not specifically associated with UPEC (fig. 15.11). The FimH adhesin at the tip of type 1 fimbriae binds a variety of host structures including Tamm Horsfall protein and uroplakins that are found on the surface of the bladder. Binding is critical to colonization of the bladder as it prevents flushing of the bacteria (reviewed by Dhakal et al. 2008). In contrast to type 1 fimbriae, P fimbriae are highly associated with UPEC, compared with fecal *E. coli*. P fimbriae are associated with pyelonephritic strains of human UPEC, but canine UPEC isolates usually carry an adhesin of P fimbriae (PapGIII) which is common among cystitis isolates and mediates binding to globopentaosylceramide (GbO_5). The genes for both type 1 and P fimbriae undergo phase variation, a reversible on and off expression, but little is known about the factors that affect their expression *in vivo*. Adherence of P fimbriae to epithelial cells involves a Galα(1–4)Galβ-containing glycolipid receptor and Toll-like receptor (TLR)-4 as coreceptor. Colonization of the bladder results in an inflammatory response

mediated by IL-6, IL-8, and other cytokines. This response is induced by TLR-4 signaling pathways activated by adherence of type 1 and P fimbriae (Bergsten et al. 2005). Interestingly, uroepithelial cells lack CD14, and therefore respond poorly to LPS. Neutrophils that are attracted to the site ingest and kill UPEC. Almost nothing is known about the role of S fimbriae in UTI.

Six iron-acquisition systems that have been identified in UPEC likely play roles in their survival and multiplication in the iron-poor environment of the bladder (Wiles et al. 2008). These systems involve siderophores (enterobactin, salmochelin, aerobactin, yersiniabactin), an ABC iron-manganese transport system (sit), and a hemin uptake system. This arrangement may provide redundancy and/or specialization for a variety of environments. For example, lipocalin 2 produced by activated neutrophils can counteract the effects of enterobactin by binding to it but is ineffective against the variant of enterobactin known as salmochelin.

Alpha-hemolysin is a pore-forming toxin that at high concentrations lyses cells, releasing nutrients. At sublytic concentrations which are likely achieved *in vivo*, alpha-hemolysin modulates host signalling pathways that may affect host cell cycling, apoptosis, and inflammation. Recently, alpha-hemolysin was reported to induce hemorrhage and sloughing of the uroepithelium in the bladder in the mouse model of ascending UTI (Smith et al. 2008). CNF1 has been reported to induce uptake of bacteria by host cells and apoptosis of epithelial cells in the bladder that could lead to exfoliation and exposure of the underlying cells. CNF1 causes inflammation and submucosal edema in the bladder of experimentally infected mice (Smith et al. 2008). Vat (vacuolating autotransporter toxin) and Sat (secreted autotransporter toxin) are serine proteases that are highly cytotoxic. Little is known about the role of Vat in pathogenesis of UPEC, but Sat causes severe kidney damage in the mouse model of ascending UTI.

UPEC appear to be better equipped to grow in urine than are non-UPEC strains. Factors possessed by UPEC that might contribute to survival in urine include resistance to the bactericidal effect of complement which is associated with certain types of capsule, O polysaccharides, specific outer membrane proteins, and aerobactin. Catheterization, abnormalities of the urinary tract that impair voiding, illnesses such as diabetes, and chemotherapy predispose to infection with a wide variety of opportunistic bacteria, including *E. coli* that lack specific urovirulence factors.

Invasion of bladder epithelial cells by UPEC is a major contributor to persistence of cystitis (Dhakal et al. 2008). FimH adhesin at the tip of type 1 fimbriae is critical for adherence and subsequent invasion of bladder epithelial cells by UPEC. FimH binds to α3 and β1 integrins on the cell surface, activating signaling cascades that cause localized changes in the cell cytoskeleton resulting in a zipper-like mechanism of entry of the bacteria into the epithelial cell. In the large terminally differentiated cells lining the bladder, the bacteria escape from the vacuoles and replicate leading to formation of intracellular bacterial communities, which can number in the thousands (Wiles et al. 2008). In immature underlying epithelial cells, UPEC localize in acidic membrane-bound vacuoles, assume a quiescent state and are likely protected to some degree from host immune surveillance and from certain antibiotics. These infected epithelial cells may persist for long periods and be the source of recurrence of a clinical UTI.

Interestingly, UPEC use both adherence and motility in the infection process. Flagella appear to contribute to the ascent of UPEC from the bladder to the kidneys, and P fimbriae synthesis has been shown to regulate motility.

Infection is cleared by exfoliation of infected bladder epithelial cells, which are primed to exfoliate, and by host defense mechanisms including neutrophils, reactive nitrogen and oxygen species, and other antibacterial compounds that are produced.

In one study, most hemolytic *E. coli* from dogs with diarrhea were shown to possess virulence gene profiles identical with those associated with UTI (Starcic et al. 2002), and the authors suggested that these may function as agents of both diarrhea and UTI. However, given that the intestine is a reservoir for UPEC and that the UPEC isolate from a dog with cystitis is frequently the same as the dominant fecal isolate (Low et al. 1988), it will be necessary to demonstrate that these *E. coli* can indeed induce diarrhea in dogs. Finally, ExPEC with virulence profiles similar to those associated with UTI are being increasingly implicated as a cause of acute hemorrhagic pneumonia in dogs.

Pyometra

Pyometra, a condition in which there is an accumulation of pus in the uterus, is the most common

genital tract infection in bitches. The pathogenesis of pyometra is not well understood, but the disease appears to develop as a sequel to a primary dysfunction involving the effect of hormone on epithelial cells of the uterus. Experimentally, bitches are most susceptible during the early stages of metestrus, when serum progesterone levels are high. The alterations in epithelial cells allow bacteria to adhere and multiply. *E. coli* is the bacterium most frequently implicated and is isolated from 58% to 88% of cases (Jarvinen 1981; Fransson et al. 1997). The *E. coli* recovered from the uterus of bitches with pyometra are usually hemolytic and have the same characteristics as UPEC (Wadas et al. 1996); in some cases there is simultaneous UTI and pyometra involving the same organism (Hagman and Kuhn 2002; Siqueira et al. 2009). These *E. coli* show a high prevalence of urovirulence genes, notably *pap*, *cnf1*, *hlyA*, *sfa*, and *fim* (Chen et al. 2003; Siqueira et al. 2009).

Pathogenic *E. coli* that reside in the intestine contaminate the vagina and ascend through the cervix into the uterus, where they adhere and multiply if there is a receptive environment. Such an environment is present in dogs in which cystic endometrial hyperplasia has developed as a consequence of cycles of exposure to estrogen and progesterone. There is a strong purulent response to the infection, a marked antibody response to the high concentration of bacteria, and systemic effects attributed to absorbed *E. coli* LPS. Both direct effects of LPS in the blood and antigen–antibody complexes in the kidney glomeruli are considered to account for renal damage and dysfunction seen in many cases of pyometra. Thus, polyuria and polydipsia are frequently seen in affected dogs.

Mastitis

E. coli mastitis can develop in any host species but is most common in dairy cattle, in which the disease is more common in high-producing animals, in the first 2 weeks after calving, and in animals with low somatic cell counts. Infection of the mammary gland by *E. coli* results in mild to severe inflammation and a clinical course of mastitis which may be peracute, acute, or chronic. The *E. coli* that are implicated in mastitis are environmental *E. coli* that do not appear to possess any special virulence factors (Wenz et al. 2006). Not surprisingly, host factors play a major role in determining the nature of the mastitis which develops in response to infection by this environmental pathogen. However, recent investigations indicate that some *E. coli* that cause mastitis have attributes similar to those of contagious mastitis pathogens, as indicated by persistence in the gland, recurring infections of the same quarter with the same genotype of *E. coli*, and apparent transfer of infection between quarters. Some *E. coli* appear to have evolved to adapt to the bovine mammary gland. One aspect of this adaptive behavior is an ability to invade mammary cells, by an endocytic pathway that avoids lysosomal degradation, thereby evading the immune system and promoting persistence in the gland (Passey et al. 2008).

Environmental *E. coli* that contaminate the teat orifice make their way through the streak canal into the gland lumen, where the host response determines the clinical outcome. Cows are most likely to become infected when they lie in feces-contaminated bedding after they have been milked and the teat orifices remain open. Experimentally, as few as 60 bacteria are sufficient to cause mastitis. The response to the presence of *E. coli* in the gland varies remarkably depending on the stage of lactation. During the dry period, a robust defence often results in elimination of the organism with little or no disease; this has been attributed to iron limitation imposed by a high concentration of the iron-binding protein lactoferrin. By contrast, in the periparturient period, the cows are in a state of immunosuppression, have a relatively low concentration of lactoferrin, and mount a delayed PMN leukocyte (PMN) response. Other factors that have been suggested to be responsible for the severe clinical manifestations seen in early lactation are a reduction of oxygen tension by bacterial growth and a resulting decrease in killing of the *E. coli* in phagolysosomes, and a rapid shift from the PMN to a less effective mononuclear leukocyte cell population in the udder. These factors permit the *E. coli* to grow to large numbers.

LPS appears to be the major bacterial factor responsible for inducing the local inflammatory response as well as a toxemia, which can be fatal. The inflammatory response is accompanied by substantial tissue damage as the short-lived neutrophils die and release toxic oxidants that kill bacteria and damage tissue. However, factors other than LPS are important as mastitis can be induced in mice that have mutated TLR-4 which consequently do not respond to LPS. Experimental infections have been useful in allowing careful measurements of local

and systemic response to infection. Increases in the concentrations of the acute phase proteins haptoglobin and amyloid-A in serum and milk have been reported and in severely affected animals increases in serum TNF-α and nitrite/nitrate have been noted.

The response to invasion of the lumen of the mammary gland by *E. coli* may be influenced by the extent of adaptation of the *E. coli* strain, the overall immune responsiveness of the host, and the concentration of opsonizing antibodies in the gland secretion. Adapted strains may induce a mild response; cows that are genetically programmed to be high immune responders may be more effective than low responders in eliminating the bacteria; and the presence of opsonizing antibodies will promote phagocytosis and killing of the *E. coli*.

The following events occur in response to infection of the mammary gland with *E. coli*. Whole bacteria or LPS stimulate the release of cytokines and other proinflammatory mediators, notably TNF-α, IL-1β, IL-8, platelet activating factor and intercellular adhesion molecule 1, which result in the migration of PMN through the connective tissue into the lumen of the gland. An increase in expression of CD44 on the PMN has been reported, but its role in disease is unclear. If the PMN response is rapid, the bacteria may be quickly phagocytosed and destroyed. Cows with ketosis have elevated serum and milk levels of β-hydroxybutyrate which may lead to neutrophil dysfunction and more severe mastitis. Failure to rapidly remove the *E. coli* results in their growth and severe local reaction and sometimes toxemia. Death may be due to endotoxemia.

In peracute disease, the animal may die without any signs of illness, but commonly there is severe toxemia as well as inflammation of the mammary gland and agalactia. In acute and subacute mastitis, there is severe to moderate inflammation of the mammary gland.

IMMUNITY

Protective immunity to enteric *E. coli* infections is based on the presence in the intestinal tract of antibodies to surface antigens. The most widely investigated of these antibodies are anti-fimbriae antibodies, which are protective when they are delivered in sufficient quantity to the intestine of nursing pigs or calves through colostrum and milk of vaccinated dams (Isaacson 1994). The antibodies may also be delivered in the feed of weaned animals (Imberechts et al. 1997a; Fairbrother et al. 2005). Antibodies to the polysaccharide capsule of ETEC may also be protective. Anti-fimbriae and anti-K antibodies function by preventing attachment of the ETEC to enterocytes.

Whereas much success has been achieved in protecting nursing animals through vaccination of the dam, the loss of milk antibodies renders weaned animals susceptible to infection. Sow's milk frequently contains antibodies to surface antigens of pathogenic *E. coli* and its addition to the diet of weaned pigs reduces the excretion of hemolytic *E. coli* including EDEC (Salajka et al. 1975; Deprez et al. 1986). In the case of ED, anti-Stx2e antibodies in the serum of pigs may also affect their susceptibility, and vaccination of pigs at 1 and 3 weeks of age with an Stx2e toxoid, or administration of antiserum against the toxoid is effective in protecting pigs (Johansen et al. 1997). Oral vaccination of pigs with a live F18-positive EDEC or with the F18 receptor conjugated to K88 (F4) fimbriae inhibits colonization of the pig intestine by F18-positive *E. coli* (Sarrazin and Bertschinger 1997).

There are differences in kinetics of infection and immunity in weaned pigs infected with K88-positive and F18-positive ETEC (Verdonck et al. 2002). Infection with K88-positive ETEC resulted in intestinal colonization and induction of anti-K88 antibodies that was more rapid than comparable events for F18-positive ETEC. Approaches to vaccination of weaned pigs against ETEC include oral immunization with live nontoxigenic ETEC, fimbrial antigens (Cox et al. 2002), or Salmonella that express K88 (F4) antigen (Lee et al. 2001).

Immune surveillance through TLRs is active in the bladder and recognition of LPS by TLR-4 is important in the innate immune response that occurs in the early stages of UTI with *E. coli* (Schilling et al. 2003). Low levels of expression of TLR-4 and defects in TLR-4, which recognizes LPS, are related to recurrence of UTIs in humans. Experimentally, there is an approximately 10-fold increase in invasion of the bladder of TLR-4 mutant mice compared with wild-type mice. Bladder epithelial cells have a rapid response pathway to LPS, mediated by cAMP, as well as the classical pathway involving NF-κB (Song and Abraham 2008). Both pathways stimulate the release of proinflammatory cytokines, notably IL-6 and IL-8. TLR-4 also appears to be capable of inducing the expulsion of intracellular UPEC.

Following infection of the urinary tract in mice, both specific anti-UPEC IgG antibodies in serum

and urine and T cells contribute to protection. Vaccination has also been shown to induce protective immunity. Administration of FimH (type 1) adhesin or PapG (P fimbrial) adhesin into the bladder has induced a protective response in a monkey model of human UTI. FimH antibodies can prevent adherence and aggregate bacteria.

Control of *E. coli* mastitis is based primarily on reducing contamination of the udder, but a bacterin consisting of killed *E. coli* strain J5, a rough mutant of an O111 *E. coli*, also provides protection against severe mastitis. The mechanism of protection has been suggested to be induction of antibodies against antigens in the core region of LPS, which is common to a large number of *E. coli*. An alternative explanation is that the vaccine improves the diapedesis of PMN into the infected gland by inducing a strong T-helper 1 response involving memory cells in the gland (Dosogne et al. 2002). Other vaccines based on the core LPS of an Enterobacteriaceae have also been reported to reduce the severity and incidence of bovine *E. coli* mastitis.

Vaccination of cattle with FecA, the receptor for the ferric citrate iron acquisition system, resulted in a reduction in clinical severity of experimentally induced mastitis (Takemura et al. 2002) and in IgG that reduced uptake of iron by *E. coli* in a reduced-iron environment (Takemura et al. 2003).

Septicemic disease in calves is most commonly the result of of agammaglobulinemia or hypogammaglobulinemia due to a failure in passive transfer of colostral immunoglobulins. It appears that specific antibodies are not required for the protection that derives from adequate immunoglobulin levels in the calf. Cross-reacting antibodies against LPS core may be a protective factor in colostrum. There is considerable evidence that IgM antibodies are the important class of antibodies that are involved in protection, likely by both antiendotoxic effects and antibacterial effects mediated by complement and antibody.

CONCLUSIONS

The spectrum of *E. coli* diseases is impressive for its range of host species and variety of mechanisms. This versatility is founded on considerable opportunity and facility in acquiring new blocks of DNA. Much has been learnt about these horizontally transmitted genes that are major contributors to virulence, but the complexity of *E. coli* pathogens is becoming evident from new data such as the genome sequences of various pathogenic *E. coli* including O157:H7. We know almost nothing about the factors which allow one particular serotype of a pathogenic *E. coli* to dominate its class, as is the case with O157:H7 among EHEC and O149:K88ac among porcine ETEC. Fitness genes that contribute to the survival of these organisms in the environment outside the host and/or to competition with other bacteria in the intestine may well be important in this regard. This should be an area of research focus to address the dearth of understanding.

E. coli will continue to evolve at a rapid pace, with selection determined by human and animal lifestyles. Pathogenic *E. coli* have shown remarkable specificity with respect to the host in which they cause disease, but recent studies have demonstrated significant similarities between human and animal ExPEC. Continuing research in this area is needed to clarify host species specificity and to assess whether *E. coli* is evolving to increase its host range and versatility even further.

There has been a rediscovery of the importance of innate immunity, and stimulation of innate immunity is a strategy that is likely to be explored further in inducing protection against some *E. coli* infections. For example, it has been suggested that intranasal administration of nontoxic inducers of TLR-4-associated antimicrobial activities may be effective in stimulating protective SIgA against UPEC in the bladder. In the face of increasing resistance of pathogenic *E. coli* to antimicrobials, alternative approaches for prevention and treatment of *E. coli* diseases of animals are sorely needed.

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16
Yersinia

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INTRODUCTION

Yersinia species cause a variety of diseases in mammals, birds, and fish. *Yersiniae* are named after Alexandre Yersin who first identified *Yersinia pestis*, the causative agent of bubonic and pneumonic plague, in 1894. For many years, the bacteria were classified as *Pasteurella*; however, they were given a separate name in 1970 as their genetic relatedness to *Enterobacteriaceae* became apparent. Like other *Enterobacteriaceae*, *Yersinae* are facultative, oxidase-negative, gram-negative rods or coccobacilli capable of fermenting glucose. *Yersinia* frequently exhibit bipolar staining in tissue smears. In the laboratory, *Yersinia* form single colonies after growth at room temperature for 2–3 days.

Of the 11 species of *Yersinia*, four, *Y. pestis*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and *Yersinia ruckeri*, cause disease in mammals, birds, and/or fish. *Y. enterocolitica* and *Y. pseudotuberculosis* are enteric pathogens that infect a wide variety of mammals and birds and generally cause syndromes ranging from mild diarrheal disease to systemic infection. Transmission generally occurs after ingestion of food or water that has been exposed to fecal matter containing these enteric pathogens (fig. 16.1). Both pathogens can survive outside of a host for a long period of time. In contrast, *Y. pestis*, the causative agent of bubonic and pneumonic plague, is generally transmitted intradermally from animal to animal via the bite of an infected flea (fig. 16.1). Occasionally transmission occurs via aerosolization from an animal or human with pneumonic plague, resulting in an often fatal pneumonia, or orally from a dead to a live animal by ingestion of contaminated carcasses. *Y. pestis* has been classified as a Category A pathogenic agent by the U.S. government, because pneumonic plague is frequently fatal in humans even with medical treatment, and *Y. pestis* was reportedly developed as a biological weapon in the former Soviet Union (Alibek and Handleman 1999). The fish pathogen, *Y. ruckeri*, often exists undetected in fish populations, particularly in rainbow trout, until stresses precipitate a disease outbreak. The virulence mechanisms and disease pathogenesis of *Y. ruckeri* will be discussed in this chapter separately from the other pathogenic *Yersinia* sp.

CLASSIFICATION OF *YERSINIA* SPECIES

Yersinia pseudotuberculosis is divided into 14 serotypes based on the immunoreactivity of its O antigens. Serotypes I-V contain strains that are pathogenic to humans and farm animals, while serotypes V1-XIV have been found in the environment and in animals, but they are not associated with clinical symptoms. *Y. enterocolitica* has been divided into five major biovars and many serotypes. Most recently serotypes 0:1 0:2, 0:3, 0:5, 0:8, and 0:9 have been associated with disease in farm animals and humans. *Y. pestis* strains are generally classified as Antiqua, Medievalis, or Orientalis, named for the three *Y. pestis* pandemics and the strains thought to have caused these three pandemics.

Although *Y. pseudotuberculosis* and *Y. enterocolitica* are both enteric pathogens, they are much less closely related at the genome level than *Y. pestis* is with *Y. pseudotuberculosis*. In fact, *Y. pestis* is

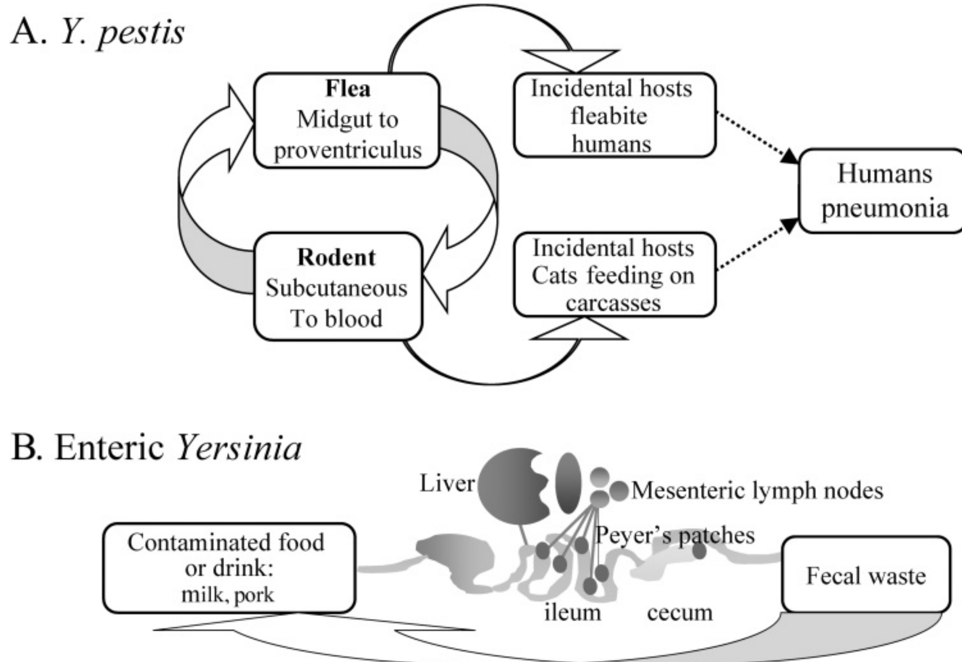

Figure 16.1. A comparison of transmission cycles of (A) *Y. pestis* and (B) the enteric *Yersinia* spp. *Y. pestis* life cycle involves transmission between fleas and rodents with the bacteria inoculated subcutaneously into rodents, eventually spreading to blood, followed by ingestion by a flea. *Y. enterocolitica* and *Y. pseudotuberculosis* are transmitted by ingestion of contaminated foods.

thought to have evolved from a *Y. pseudotuberculosis* serotype Ib strain within the last 20,000 years and possibly as recently as 1500 years ago (Achtman et al. 1999). In contrast, *Y. enterocolitica* and *Y. pseudotuberculosis* are thought to have diverged from a common ancestor about 5 million years ago. Because of the high degree of DNA homology between *Y. pestis* and *Y. pseudotuberculosis*, *Y. pestis* could be considered a subspecies of *Y. pseudotuberculosis*. However, the designation "*Y. pestis*" is still used because of the distinct and drastic differences between *Y. pestis* and *Y. pseudotuberculosis* in their ability to survive in the environment, in the spectrum and severity of disease, in their mode of transmission, and in some of their virulence factors.

Recently, the DNA sequences of several *Y. pestis* strains and a *Y. pseudotuberculosis* strain have become available. A comparison between the *Y. pestis* and the *Y. pseudotuberculosis* genomes indicates that the *Y. pestis* genome has been in rapid flux since its divergence from *Y. pseudotuberculosis* and has lost many genetic elements while undergoing genomic rearrangements. In addition, *Y. pestis* has acquired two plasmids, pFra and pPCP1, which are absent in *Y. pseudotuberculosis* strains. As will be discussed here, these plasmids encode proteins that enable *Y. pestis* to survive in the distinct niches it occupies, such as growth in the flea and spread from subcutaneous inoculation sites. Interestingly, some of the lost genes are important for the virulence and/or transmission of enteric *Yersinia* sp., while others are likely needed by the enterics while growing in the environment. In all, *Y. pestis* has acquired around 30 genes in addition to the genes encoded on its two unique plasmids since it diverged from *Y. pseudotuberculosis*, while *Y. pseudotuberculosis* has over 500 genes that are either not found or no longer expressed in *Y. pestis* (Chain et al. 2004; Pouillot et al. 2008). Plasmids, pathogenicity islands, and proteins expressed in the 11 species of *Yersinia* are shown in table 16.1.

Table 16.1. Plasmids, Pathogenicity Islands, and Proteins Expressed in Various *Yersinia* spp.

Y. pestis		pYV	pPCP	pFra	HPI	HMS	Ysa	Inv[c]	YadA[c]
	Antiqua	+	+	+	+	+	+	–	–
	Medievalis	+	+	+	+	+	+	–	–
	Orientalis	+	+	+	+	+	+	–	–
Y. pseudotuberculosis									
	I	+	–	–	+	+	+	+	+
	II	+	–	–	–	+	?	+	+
	III	+	–	–	(+)[a]	+	+	+	+
	IV	+	–	–	–	+	?	+	+
	V	+	–	–	–	+	?	+	+
Y. enterocolitica									
	1a	–	–	–	–	–	–	?	–
	1b	+	–	–	+	–	+[b]	+	+
	2	+	–	–	–	–	–	+	+
	3	+	–	–	–	–	+[b]	+	+
	4	+	–	–	–	–	–	+	+
	5	+	–	–	–	–	?		+
Y. ruckeri		–	–	–	–	–	?		–
Yersinia intermedia		–	–	–	–		–		–
Yersinia frederiksenii		–	–	–	–		–		–
Yersinia kristensenii		–	–	–	–		–		–
Yersinia aldovae		–	–	–	–		–		–
Yersinia rhodei		–	–	–	–		?		–
Yersinia mollaretti		–	–	–	–		–		–
Yersinia bercoviere		–	–	–	–		–		–

[a]The HPI contains a 9-kb truncation in *Y. pseudotuberculosis* serotype III.
[b]*Y. enterocolitica* contains a different type III secretion system on the chromosome from the systems found in *Y. pestis* and *Y. pseudotuberculosis*.
[c]The DNA for the *inv* and *yadA* genes is present but the proteins are not made because of an insertion element and frameshift mutation in the two genes.

SOURCES OF *YERSINIA* SPECIES

The enteric *Yersinia* pathogens are usually acquired through ingestion of contaminated food or water. Infection in humans is often associated with eating undercooked or raw pork, although recent outbreaks in humans have occurred after ingestion of contaminated milk products or fresh produce. Birds and rodents are also reservoirs of infection for *Y. pseudotuberculosis* and may contaminate feedstuffs. Swine are a significant source of *Y. enterocolitica* and may infect ruminants if the two species share adjacent enclosures. The enteric *Yersinia* sp. replicate at temperatures as low as 4°C, so refrigeration does not make contaminated food safe. Since enteric *Yersinia* can spend significant portions of their life outside of the host in environments with varied sources of nutrients, they retain metabolic capabilities that have been lost in *Y. pestis*.

Fleas, in particular the rat flea, *Xenopsylla cheopis*, are an efficient arthropod vector of *Y. pestis*, the plague bacillus. Transmission occurs after a flea feeds on an infected host. The flea ingests the blood meal containing some bacilli and the bacilli incubate in the midgut of the flea. Over the course of several weeks, the bacteria replicate and form a clot that lodges in the proventriculus of the flea. The proventriculus is a valve between the esophagus and midgut. Clot formation in the proventriculus blocks ingestion of blood, and thus

prevents normal feeding by the flea. The inability to feed properly prompts the flea to regurgitate the bacterial clot into a host prior to ingestion of blood and eventually kills the flea. Thus a mammal becomes intradermally inoculated with the plague bacilli. In order for the cycle of transmission to be completed, an infected animal or person must become bacteremic so that a feeding flea ingests some bacilli. Indeed in many infected animals high levels of bacteria are found systemically and thus fleas feeding on a terminally infected host or dead carcasses are likely to ingest the bacillus. *Y. pestis* may also enter through skin lesions or orally if a human or animal comes into contact with an infected animal or carcass. *Y. pestis* can survive for weeks to months in organic material, for example, carcasses, but is otherwise sensitive to desiccation and temperatures above 40°C. Colder temperatures or freezing may actually prolong the viability of the organism.

VIRULENCE FACTORS

Factors Necessary for *Yersinia pestis* Life Cycle in the Flea

Two genes that are present in *Y. pestis*, but not the enteric *Y. pseudotuberculosis*, enable *Y. pestis* survival within the flea midgut and transmission from the flea to the human host. In the 1950s, a pMT plasmid-encoded Ymt protein was characterized as a *Yersinia* murine toxin because it had β-adrenergic blocking ability in mice and rats but not other animals. When Ymt is released from *Y. pestis* in the late stages of septicemia, hypotension and vascular collapse occur. However, lethality of *Y. pestis* in mice is not dependent on Ymt, which is expressed at higher levels at 26°C compared with 37°C; these observations prompted a recent study of Ymt in fleas. That study showed that *Y. pestis* strains that lack Ymt are rapidly killed in the midgut of fleas. Ymt is a phospholipase D (PLD) that remains within the bacteria unless the bacteria lyse (Hinnebusch et al. 2000, 2002). How PLD protects *Y. pestis* from bactericidal components in the flea mi-gut and what in the midgut is bactericidal to *Y. pestis* are not known. Nonetheless, without Ymt, *Y. pestis* is severely impaired in its ability to survive in one crucial niche in its life cycle.

A second gene locus, hemin storage (*hms*) locus, is necessary for efficient transmission of *Y. pestis* from the flea to intradermal sites (Hinnebusch et al. 1996). Normally, *Y. pestis* will form a clot in the midgut and within a week the bacteria migrate to the proventriculus. Clot formation in the proventriculus is dependent on the *hms* genes. Bacilli lacking these genes can still replicate in the flea midgut but do not lodge en masse in the proventriculus and thus the bolus is not transmitted efficiently when the flea feeds on a host.

The High Pathogenicity Island and Iron Acquisition Mechanisms

The acquisition of iron by *Y. pestis* is critically important for virulence. *Y. pestis*, *Y. pseudotuberculosis* serotypes O1 and O3, and *Y. enterocolitica* biotype 1B strains contain a 35–42 kb chromosomal element, called the High Pathogenicity Island (HPI) that contains genes enabling iron acquisition (reviewed in Carniel 2001). Mutations of the HPI locus render *Y. pestis* significantly less virulent after subcutaneous inoculation, and the presence of this locus correlates with the level of pathogenicity of enteric *Yersinia* sp.; both observations support the notion that this segment of DNA is required for high pathogenicity. Similar DNA sequences have also been identified in some *Escherchia coli* strains isolated from patients with diarrhea and in some strains of *Salmonella*, *Citrobacter*, and *Klebsiella*. The HPI was likely acquired by *Yersinia* sp. from another organism based on three observations: its guanine:cytosine (GC) base composition differs from the rest of the genomic sequence; it is flanked by insertion sequence elements, IS100; and it encodes a putative integrase, all features that are hallmarks of horizontally transmitted elements. Furthermore, different *Yersinia* sp. likely acquired the HPI at independent times because the HPI is located within different tRNA genes at different chromosomal locations. In *Y. pestis*, the HPI is adjacent to another mobile genetic element, the *hms* locus discussed above, which suggests that this chromosomal region is particularly susceptible to invading DNA elements for recombination.

The HPI contains 11 genes that are involved in iron uptake. Of central importance is the siderophore, yersiniabactin, which binds to Fe^{3+} with a higher affinity than Fe^{2+}. In general, siderophores scavenge Fe^{3+} bound to host proteins and transport it back to the bacteria. Once within the cytoplasm the Fe^{3+} can be used for the metabolic functions of the bacteria. Yersiniabactin is synthesized in a ribosome-independent fashion by five proteins, which are encoded by genes found on the HPI. In addition

to the outer membrane receptor for yersiniabactin, two inner membrane permeases that escort Fe3+ into the cytoplasm of the bacteria and proteins required for synthesis of this system are encoded on the HPI.

Y. pestis has additional iron acquisition mechanisms, including the siderophore-independent processes involving ferric uptake controlled by Yfu and Yfe (e.g., see Perry et al. 2003).

Fraction 1 Antigen

Fraction 1 antigen (F1) is associated with the capsule and is encoded on the p60 or pFra plasmid found only in *Y. pestis*. F1 can prevent opsonization by complement and thus F1 production is one of the several mechanisms utilized by *Y. pestis* for evading phagocytosis by neutrophils and macrophages.

Plasminogen Activator Pla

Pla is encoded on the pPCP1 plasmid found in *Y. pestis*. Pla is required for *Y. pestis* to disseminate from an intradermal site of inoculation to surrounding lymph tissues or the bloodstream (Sodeinde et al. 1992). Pla is an outer membrane protein that has proteolytic activity, activates plasminogen, and acts as an adhesin. Its proteolytic activity presumably is responsible for the digestion of the connective tissue surrounding the site of inoculation. Thus, acquisition of pPCP1 must have been necessary for *Y. pestis* to evolve from an enteric pathogen, which establishes a site of infection in the gastrointestinal tract to one that establishes infection after intradermal inoculation. In addition, Pla facilitates invasion of *Y. pestis* into epithelial cells and human endothelial cells, an activity distinct from its proteolytic activity (Lahteenmaki et al. 2001). Nonetheless, its ability to promote cellular invasion may also aid in the initial dissemination of *Y. pestis* from its intradermal location to the lymph nodes or other organs. Although previously shown to be required for bubonic plague, recent research found that Pla-negative *Y. pestis* is partially defective for dissemination from the lungs of mice in a pneumonic plage model (Lathem et al. 2007). Thus, Pla is important during multiple stages of *Y. pestis* pathogenesis.

Adherence and Invasion Factors

A number of adherence factors have been identified in *Yersinia* sp., but curiously several of the adherence factors found in the enteric *Yersinia* sp. have been inactivated in *Y. pestis* by insertion of insertion sequence (IS) elements or frameshift mutations. This observation suggests that the inactivated adherence factors are not necessary for the relatively different lifestyle of *Y. pestis* or that *Y. pestis* has acquired additional adherence factors. Five adhesins of note are the chromosomally encoded invasin (Isberg and Falkow 1985), Ail (Miller and Falkow 1988), the pH 6 antigen (Makoveichuk et al. 2003), the pYV-encoded YadA, and pPCP1-encoded Pla. Invasin, YadA, and Ail are expressed in the enteric *Yersinia* sp.; pH 6 antigen is found in both *Y. pestis* and *Y. pseudotuberculosis* and Pla in *Y. pestis*. Analysis of the *Y. pestis* genome indicates that several putative genes encode proteins that have homology to invasin or other adherence factors; however, no studies have been done to demonstrate that these putative proteins function as adhesins and/or are important in virulence.

Invasin is the best characterized adherence and invasion factor in enteric *Yersinia* sp. It binds avidly to $\alpha\beta1$ integrins found on the surface of many types of mammalian cells. This tight binding triggers normally non-phagocytic cells to internalize *Yersinia*, if the Yops are not being expressed (see below). One role of invasin in enteric *Yersinia* pathogenesis appears to be to allow the pathogen to survive in the gastrointestinal tract. *Y. pseudotuberculosis* strains that lack invasin are rapidly cleared from the gastrointestinal tract, which suggests that invasin might be critical for efficient dissemination of *Yersinia* from one animal to another. Expression of invasin in mouse model systems of infection also correlates with the ability of enteric *Yersinia* sp. to rapidly invade the Peyer's patches lining the small intestine after oral inoculation (Pepe et al. 1995; Marra and Isberg 1997).

In *Y. enterocolitica*, YadA and Ail provide resistance to the bactericidal effects of serum in addition to their ability to promote adherence and invasion into mammalian cells (Bliska and Falkow 1992). After oral inoculation of mice in a model system of infection, *yadA* mutants fail to persist in lymph tissues; this finding demonstrates the importance of YadA for pathogenesis of *Y. enterocolitica* (Pepe et al. 1995).

Type III Secretion System

One of the key virulence features of *Yersinia*, a type III secretion system, is encoded on a 70-kb virulence plasmid found in *Y. enterocolitica*, *Y. pseudotuberculosis* (called pYV), and *Y. pestis* (called pCD1).

Type III secretion is a specialized system that allows a bacterium to translocate "effector" proteins, called Yops in *Yersinia*, from the bacterial cytoplasm into host cells, thus this system transports proteins across three membranes (reviewed in Cornelis 2002). Once within host cells, these proteins alter, neutralize, or destroy normal host cell functions. A number of other gram-negative animal, fish, and plant pathogens, including *Salmonella enteriditis*, *Bordetella*, and *Pseudomonas*, contain type III secretion systems (Hueck 1998); however, the *Yersinia* pYV encoded system was one of the first identified and is one of the best characterized type III systems.

Structurally, the type III secretion apparatus resembles flagellar apparatuses found in bacteria. In fact, many of the genes encoding the flagellar basal body and type III secretion apparatus share sequence similarity. Around 25 proteins comprise the structural components of the type III secretion system that spans from the cytoplasm to the outer membrane and includes a needle-like structure extending beyond the outer membrane, through which exported proteins presumably travel. In *Yersinia* sp., the type III secretion system is regulated by temperature. At cool and ambient temperatures, the type III secretion system is not highly expressed and little secretion of the Yops is detected; however, after shift to 37°C, expression of the genes encoding the secretion apparatus and Yops increases. VirF, a DNA-binding protein, positively regulates the induction of synthesis in response to temperature (Yother et al. 1986), while the chromosomally encoded Ymo negatively regulates Yop expression (Lambert de Rouvroit et al. 1992).

Yops are translocated into a mammalian cell when a bacterium binds to it, via either one of the adhesions, such as invasin or YadA, or when the bacterium is opsonized by IgG or complement. Several components of the type III secretion system are necessary for translocation of Yops into host cells, but not for secretion of Yops into the extracellular milieu. Two proteins, YopB and YopD, form a pore in the mammalian cell membrane through which the Yops are thought to travel. In addition, LcrV is required for Yop translocation into mammalian cells and is found on the tip of the needle of the type III secretion apparatus (Mueller et al. 2005). This location and the fact that LcrV is essential for pore formation, but is not itself found in the mammalian host cell membrane, suggest that LcrV facilitates assembly of the pore.

Historically, the observation that *Yersinia* grown in medium with low concentrations of calcium secrete Yops regardless of the presence of host cells and do not require YopB or YopD for secretion was very useful for researchers in identifying Yops and components of the type III secretion system. However, it remains unclear if the so-called "low calcium response" has any relevance to Yop delivery in an infected animal since physiological levels of calcium are generally high. In fact, it is generally thought that Yops are translocated directly into host cells in a polarized fashion from bacterial cytosol to mammalian cytosol during infection and that only very low levels leak into the extracellular milieu.

In addition to the plasmid-encoded type III secretion system, the three pathogenic *Yersinia* species contain a second type III secretion system encoded on the chromosome (Foultier et al. 2002). Curiously, the chromosomal type III secretion system found in *Y. enterocolitica* differs from those found in *Y. pestis* and *Y. pseudotuberculosis*, suggesting that the chromosomally encoded systems were acquired independently and after the enteric *Yersinia* sp. diverged from each other. Further evidence supporting the notion that the chromosomal type III secretion systems were acquired at different times is the observation that not all *Y. enterocolitica* strains contain the chromosomal type III secretion system. Those strains that do, however, are more virulent in humans than strains that lack the chromosomal type III secretion system. Investigators are actively researching the proteins secreted by the chromosomal type III secretion system and their role in virulence in *Y. enterocolitica* (Young and Young 2002; Foultier et al. 2002). The chromosomal type III secretion system in *Y. pestis* and *Y. pseudotuberculosis* has not been studied as intensively and no role in virulence has been described for the chromosomal type III secretion system in either *Y. pestis* or *Y. pseudotuberculosis*.

Yops

While type III secretion apparatuses are highly conserved among many pathogenic bacteria, the effector proteins secreted by different pathogens often differ in function and biochemical activity (Hueck 1998). At least six Yops are translocated into host cells by the plasmid-encoded type III secretion system (Cornelis 2002). Recent work with *Y. pestis* indicated that Yops are found more frequently in cells of the innate immune system, such as

neutrophils and macrophages, rather than in B and T cells (Marketon et al. 2005). The combined functions of the *Yersinia* Yops allow the bacteria to remain extracellular and to evade the bactericidal effects of neutrophils and macrophages using a variety of mechanisms. For instance, four of the six well-studied effector Yops—YopE, YopO, YopT, and YopH—inhibit phagocytosis by macrophages and neutrophils (Grosdent et al. 2002). In contrast to the *Yersinia* Yops, effector molecules secreted by *Salmonella* induce host cells to take up the bacterium and allow *Salmonella* to grow intracellularly in host cells, highlighting the fact that different effectors from different bacteria manipulate mammalian cells in distinct ways.

Interestingly, most Yops interact with more than one protein target, suggesting that the Yops could have multiple cellular targets during infection and/or can alter multiple host processes within a single cell. YopH is a potent tyrosine phosphatase that dephosphorylates a number of proteins clustering at focal adhesion complexes after invasin binds to $\alpha\beta1$ integrins. The rapid dephosphorylation of these proteins results in the cessation of normal signal-transduction cascades that occur after receptors become engaged. In B and T cells, YopH causes inappropriate dephosphorylation of proteins that are stimulated in response to T cell receptor or B cell receptor binding to substrates (Yao et al. 1999). This observation indicates that if YopH enters B and/or T cells during infection, it could alter the normal development of these cells in response to antigen and weaken or modify specific immune responses to antigens.

Three Yops—YopE, YopT, and YopO—all cause perturbations to the actin cytoskeleton after infection and all three interact with Rho family proteins, which are involved in controlling actin cytoskeleton dynamics. YopE has GTPase activity on several Rho family proteins and thus catalyzes the conversion of the active, GTP-bound form of RhoA and Rac1 into their inactive, GDP-bound form. These activities cause the destruction of actin stress fibers and prevent macrophages and neutrophils from phagocytosing *Yersinia*. In addition, once in mammalian cells, YopE causes *Yersinia* to stop translocating Yops. This feedback mechanism may ensure that once a certain concentration of Yops is reached in host cells, the bacteria cease translocation and conserve Yops for a new cell target. YopT targets and cleaves RhoA, causing its removal from the membrane and leading to an increase in its inactive RhoGDI-bound form. YopO (also called YpkA) has serine-threonine kinase activity, binds to Rho family proteins, and inhibits signaling from Gαq proteins (Navarro et al. 2007). In addition, analysis of the YpkA crystal structure suggested that it functions as a Rho family guanidine nucleotide dissociation factor (Prehna et al. 2006). The fact that all three of these Yops are important in virulence in mice and that all target Rho family proteins to disrupt actin cytoskeleton indicates that *Yersinia* relies on multiple means to disable host defenses through targeting of small Rho family GTPases.

YopJ (YopP in *Y. enterocolitica*) has the striking ability to cause murine macrophages to undergo apoptosis (programmed cell death). However, *yopJ* mutants have only mild, if any, defects in virulence studies in mice, suggesting that YopJ plays a minor role in colonizing tissues or causing disease in these mammals. Nonetheless, it remains possible that YopJ has a significant role in virulence in a host that has not been studied experimentally, and thus it may be important in some types of infections but not others. On the other hand, a recent study demonstrated that reduced secretion of YopJ caused an increase in the virulence of *Yersinia* in mice, suggesting that it may modulate virulence in some fashion (Brodsky and Medzhitov 2008). Several biochemical functions have been ascribed to YopJ with data supporting its role as a deubiquitinating protease and a serine/threonine acetyltransferase (e.g., see Prehna et al. 2006). YopM binds to RSK, ribosomal S6 kinase, which regulates a variety of genes involved in cell cycle regulation (McDonald et al. 2003). Both RSK and YopM are found in the nucleus and cytoplasm of cells; however, how YopM functions in mammal cells and how it alters RSK functions have not been elucidated.

Other Virulence Factors

In addition to the aforementioned virulence factors a number of other genes have been shown to alter the virulence of one or more of the pathogenic *Yersinia spp*. in model systems of infection. These genes encode proteins for iron acquisition, superantigens found in some enteric strains, dam methylases, ureases, pili, and transcriptional regulators such as RovA. The interested reader can find literature on these and other factors in citations on PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez).

PATHOGENESIS

Tropism for Lymph Nodes

A hallmark of infection with *Yersinia* in mammals is that the bacteria show a marked tropism for lymph tissues. The enteric *Yersinia* sp. rapidly invade the Peyer's patches lining the small intestine and frequently cause mesenteric lymphadenitis. *Y. pestis* infects the draining lymph nodes around the area of intradermal inoculation, causing the infected nodes to swell up. These large swollen nodes are called "buboes," from which the name "bubonic" plague was derived. Although all three mammalian *Yersinia* pathogens contain the 70-kb plasmid, which is not found in *Y. ruckeri* or the seven nonpathogenic *Yersinia* sp., the presence of pYV does not completely account for the tropism for the lymph nodes, as strains lacking pYV colonize the mesenteric lymph nodes in both people and animals. Recently, a study of lymph node pathologies in mice after infection with *Y. pestis* versus *Y. pseudotuberculosis* showed that the host response to infection differed significantly. Infection with *Y. pestis* induced vascular congestion and many free bacteria were observed in the tissues, whereas infection with *Y. pseudotuberculosis* induced polymorphonuclear cell migration to the tissue that appeared to form abscesses containing the bacteria (Guinet et al. 2008).

Extracellular or Intracellular Pathogen?

The question of whether *Yersinia* sp. are primarily intracellular or extracellular pathogens when infecting a host has been discussed for several decades in the literature. Much evidence exists that *Yersinia* primarily resides extracellularly during infection, especially once the bacillus has established a fulminant infection in lymph tissues or organs, such as the spleen and the lungs. The requirement for the anti-phagocytic Yops during animal infection is also suggestive of an extracellular lifestyle. Yet there is compelling evidence that *Yersinia* acts intracellularly in mammals at certain stages of infection. For instance, rapid colonization of the Peyer's patches is dependent upon the bacterial protein invasin and occurs by the bacterium transcytosing through M cells (Marra and Isberg 1997). M cells are specialized epithelial cells that sample intestinal contents, present antigens to the underlying lymphoid tissues, and are interspersed among epithelial with microvilli. In addition, there is evidence that *Y. pestis* and its close relative *Y. pseudotuberculosis* replicate in macrophages and there is speculation that intracellular replication is important during the initial stages of infection (Straley and Harmon 1984). Finally, there is indirect, immunological evidence that enteric *Yersinia* sp. reside intracellularly because a humoral response to *Yersinia* is not sufficient to clear the infection, rather a T cell response is required (Autenrieth et al. 1993). Thus the answer as to whether *Yersinia* sp. are extracellular or intracellular pathogens may be "both" and depends upon the stage of the infection process.

Yersinia pestis Infection in Mammals

The coccobacillus, *Y. pestis*, has caused three major pandemics during the course of human history: the Justinian plague, which raged for about 50 years starting around 540 A.D., the medieval plague, or Black Death, which is estimated to have killed at least 25% of the European population in the 14th century, and the Modern Orientalis pandemic, which started in 1894 and killed about 10 million people by the early 20th century. Recent outbreaks of bubonic plague continue to occur throughout the world, including one in Algeria in 2003 and another one in the Yunan province of China in 2005.

House rats and their fleas play a significant role as plague vectors during human epidemics. A 1994 outbreak in India followed a natural disaster that led to the disruption of sanitation procedures and a boom in rat populations. It was the first outbreak in India in over 30 years and several cases of pneumonic transmission may have occurred. In crowded cities, infections cycle between rats and fleas with incidental infection of humans or other domestic animals; however, in such places the potential exists for outbreaks to occur among humans. In nature, *Y. pestis* causes sylvatic infections. Wild rodents are more often associated with the sporadic cases of human plague reported in the United States than are domesticated animals. In the United States, outbreaks in rodent populations occur in the states west of the Mississippi River. Rock squirrels and ground squirrels (*Spermophilus* sp.) and their fleas are the most common sources of human infections in these areas, whereas domestic cat and dog fleas (*Ctenocephalides* sp.) are considered poor vectors for plague. Other rodent species such as prairie dogs and chipmunks are susceptible to plague and experience mortality of up to 100% during plague outbreaks. Coyotes are often used as sentinel species in plague outbreaks in areas of sylvatic plague.

Domestic dogs, wild birds, and wild carnivores, with the exception of wild and domestic cats, are relatively resistant to plague. Dogs tend to exhibit nonspecific clinical signs and are considered less of a zoonotic threat to humans. On the other hand, cats are more susceptible to plague than are dogs and other wild carnivores and are a threat to transmit the bacillus to humans. Black-footed ferrets are also sensitive to plague. The reintroduction of this endangered species into its natural habitat has been hampered due to the widespread distribution of plague in prairie dog populations in the same area. Siberian polecats are used as a model to study the pathogenesis of plague in this endangered species.

While *Y. pestis* enters the body most frequently through fleabites, the organism may also enter through skin lesions if a human or animal comes into contact with an infected animal or carcass. Experimental and anectodal evidence indicates that cats primarily become infected after eating infected carcasses. Infected cats can transmit infection to humans through bites, scratches, or accidental ingestion of contaminated material (Gage et al. 2000). Humans exposed to respiratory aerosols from infected cats with pneumonic plague can also acquire the organism through mucous membranes, and thus veterinarians are cautioned to be mindful when handling suspected plague-infected animals. Human to human transmission occurs rarely; however, this form of transmission, which leads to pneumonic plague, is highly fatal.

Three forms of the plague exist in humans and have been documented in cats as well. If left untreated, infection in humans and some animals, including cats, is fulminant and fatal. In general, streptomycin is the antibiotic of choice for treating plague, although chloramphenicol and tetracycline can also be used; tetracyclines and sulfonamides are recommended for prophylaxis. The most common and least fatal form is bubonic plague, which has an approximately 30% fatality rate if untreated. After intradermal inoculation of the skin, *Y. pestis* disseminates to the regional lymph nodes, which become enlarged and painful. These nodes form abscesses termed buboes. When cats acquire the disease by ingesting an infected rodent, the lymph nodes in the head and neck region are frequently affected. The most frequent histopathologic finding of plague in cats is a necrosuppurative inflammation of lymph nodes with destruction of normal nodal architecture (Watson et al. 2001). Tularemia, caused by *Francisella tularensis*, may also cause lymphadenopathy in cats, thus the two diseases need to be differentiated by clinical history, culture, or lymph node aspirates.

The septicemic form of plague may occur as a sequel to the bubonic form of plague through hematogenous or lymphatic spread, or it may occur as a primary condition. Other than lymph nodes, the most frequently affected organs in septicemic plague are the spleen in people and the lungs in cats (Gage et al. 2000; Watson et al. 2001). In cats, the palatine and pharyngeal tonsils, spleen, and adrenal glands may exhibit histopathologic changes due to colonization by *Y. pestis*.

Pneumonic plague occurs as a sequel to either septicemic or bubonic forms or after intranasal or aerosol exposure. Pneumonic plague is the most fatal type of *Y. pestis* infection with greater than 90% of humans dying if untreated. Cats with pneumonic plague are a serious hazard due to the potential for persons exposed to these animals to inhale respiratory aerosols. One study indicated that 5 of the 23 cases of cat-associated human plague in the United States between 1977 and 1998 were fatal (Gage et al. 2000).

Enteric *Yersinia* Spp. Infection in Mammals

Infections of *Y. pseudotuberculosis* in humans are rare, self-limiting, and generally do not require antibiotics. In otherwise healthy individuals infection of the mesenteric lymph nodes is more common than gastroenteritis; however, people with hemochromatosis (iron overload disease) or underlying liver ailments can develop severe, potentially lethal systemic infections with *Y. pseudotuberculosis*. Infection of humans with *Y. enterocolitica* more often results in diarrhea and gastroenteritis although mesenteric lymphadenitis can occur (Metchock et al. 1991). Mesenteric lymphadenitis due to enteric *Yersinia* infections can be misdiagnosed as appendicitis. A common sequel from enteric *Yersinia* infections, particularly in people carrying the HLA B27 MHC allele, is reactive arthritis (Bottone 1997).

Yersinia pseudotuberculosis is a common inhabitant of the gastrointestinal tract of a wide range of animals, both domestic and wild, including birds, rodents, primates, hoof stock, and laboratory animals. The organism is frequently isolated from the feces of asymptomatic animals including cats, dogs, swine, and hoofstock. *Y. pseudotuberculosis* is also prevalent in poultry flocks, with turkeys and

young birds being most frequently affected. Clinical disease can range from sudden death to chronic ill thrift with persistent diarrhea. Many carnivores, including cats and dogs, are relatively resistant to clinical disease caused by *Y. pseudotuberculosis*.

Clinical disease and herd outbreaks may be precipitated by stress, such as in shipping, overcrowding, starvation, or extreme weather conditions. Most often clinical symptoms of *Y. pseudotuberculosis* infection include enterocolitis and mesenteric lymphadenitis. Occasionally *Y. pseudotuberculosis* can cause hepatic necrosis and splenitis, particularly in sheep. *Y. pseudotuberculosis* has also been implicated as the causative agent in abortions in sheep, cattle, and goats. Cases of mastitis in does and epididymitis and orchitis in rams have been reported.

Yersinia enterocolitica, like *Y. pseudotuberculosis*, causes enterocolitis in several species of animals. Sheep and goats appear to be the most frequently affected species but swine are an important reservoir for this organism. Young kids appear to be particularly susceptible to acute catarrhal enteritis, which is often fatal. As with other *Yersinia* species, carnivores do not usually develop clinical disease when exposed to *Y. enterocolitica*.

After oral ingestion, the enteric *Yersinia* migrate to the ileum, cecum, and ascending colon of the intestine and the associated lymph tissues. Infection with *Y. entercolitica* and *Y. pseudotuberculosis* causes a suppurative erosive enterocolitis in all species. Clinical disease may range from an acute, fulminant infection to a mild chronic infection. The following gross lesions may be noted throughout the intestinal tract: increased fluid of intestinal contents, small nodules on the mucosal surface, mild erosions or ulcerations, and fibrinous exudate. The jejunum and ileum are the most frequently affected segments of the intestine although the cecum and proximal colon may also be involved. Initially *Yersinia* colonization of the intestinal mucosa results in micro abscesses in the lamina propria. These micro abscesses contain aggregates of coccobacilli and neutrophils forming necrosuppurative masses, which may eventually coalesce into mucosal erosions. These micro abscesses may penetrate through the muscularis mucosa. The abscessation and infiltration of inflammatory cells causes a loss of integrity of the mucosa, atrophy of intestinal villi, and hypertrophy of crypts. As a result of mucosal damage, fluid loss occurs, resulting in diarrhea due to malabsorption.

Diarrhea tends to be non-hemorrhagic unless fulminant yersiniosis occurs. Both *Y. pseudotuberculosis* and *Y. entercolitica* can disseminate via the portal circulation and mesenteric lymph nodes to major lymphatics, and can also disseminate to liver and spleen, causing micro abscesses and acute death. Analysis of *Y. pseudotuberculosis* infection in a mouse model revealed the assumed stepwise order of dissemination, from intestine to intestinal lymphoid tissue to blood-filtering organs, to be incorrect. Analysis of clonal bacterial populations in infected tissues, coupled with observed systemic infection in mice lacking Peyer's Patches, indicated that *Y. pseudotuberculosis* spreads directly from the intestine to systemic tissues (Barnes et al. 2006). If intestinal mucosal damage already exists, for example, due to parasites, animals may be predisposed to septic yersiniosis and reproductive disease. Typical intestinal infection is marked by edematous mesenteric lymph nodes, while during severe acute infection the Peyer's patches are notably altered, with colonies of bacteria and necrotic abscesses.

Abortion, stillbirths, orchitis, and epididymitis in ruminants may occur as sequelae to bacteremia subsequent to intestinal colonization by the bacilli. *Yersinia* invades the caruncle of the uterine endothelium and the fetal cotyledons and thereby gains access to the choriollantois and fetus. Grossly, the uterus may contain purulent material or may be hemorrhagic, with some thickening of the placenta and necrotic debris in the intercotyledonary zone. A mixed infiltrate of inflammatory cells and thrombosis of placental vessels has been described. The fetus may contain thoracic and abdominal effusions and necrotic microabscesses in the liver.

Yersinia pseudotuberculosis, but not *Y. enterocolitica*, has been documented to cause mastitis in does. Mastitis occurs as a result of direct invasion of the teat canal, rather than as sequelae to bacteremia. Udders are edematous and may have clots in the milk or blood, in acute and chronic mastitis.

Yersinia ruckeri in Fish

Yersinia ruckeri is the causative agent of enteric redmouth disease in marine and freshwater fish and yersiniosis in salmonids. *Y. ruckeri* has also been isolated from other animals, including birds, otters, and humans. Enteric redmouth is a hemorrhagic inflammation of the perioral subcutis in rainbow trout and occasionally in other freshwater fish. The disease is characterized by darkening of the skin

with subcutaneous hemorrhage of the throat and mouth. Erosion of the jaw and palate may occur if the condition is left untreated. Grossly, hemorrhage and petechiation occur on the skin and in internal organs. Acute death without clinical signs is common in young fish.

Fish tend to be asymptomatic when harboring *Y. ruckeri* until external stresses cause clinical disease. Outbreaks of enteric redmouth disease are most frequently observed in intensive farming situations where stress factors are increased. Transmission of *Y. ruckeri* is by fecal shedding of the bacteria. The most significant environmental factor associated with stress is poor water quality, including an increased load of organic matter, often from overcrowding, and increased water temperatures, which results in lowered oxygen content. *Y. ruckeri* exists undetected in fish populations until these stresses precipitate a disease outbreak. Systemic infection and significant mortality can occur in fish hatcheries. Horne and Barnes (1999) provide an excellent review of *Y. ruckeri*.

Despite genetic similarity to other *Yersinia* sp., *Y. ruckeri* uses distinct virulence mechanisms (Fernandez et al. 2007). Most notably, *Y. ruckeri* lacks the plasmid-encoded type III secretion system and effector Yops. Instead, this pathogen utilizes biofilms, adherence and invasion factors, extracellular toxins, and serum resistance proteins to cause disease. Additionally, *Y. ruckeri* can survive in brackish and freshwater, thus enhancing the likelihood of transmission to new hosts. Screens to identify *Y. ruckeri in vivo* expressed genes and surface proteins have pinpointed additional virulence factors of unknown mechanism. Immersion or injection of salmonids with *Y. ruckeri* provides an animal model for testing of putative virulence factors, and fish cell lines have been used for *in vitro* assays of adherence and invasion. The existence of animal and cell culture models, coupled with the ability to genetically manipulate the bacteria, bodes well for future investigations of *Y. ruckeri* virulence strategies.

Immune Responses to *Yersinia* SP.

Yersinia replication in systemic organs, if unchecked, causes significant host morbidity and mortality. This observation indicates that *Yersinia* effectively disarms immune responses. Host responses to microbes begin with innate recognition, when Toll-like receptors (TLRs) and other host proteins bind to common microbial ligands and signal the presence of potentially inappropriate bacterial colonization. This signal activates antimicrobial functions of macrophages and neutrophils, which then destroy the offending microbe. However, *Yersinia* has evolved multiple mechanisms to compromise innate immune function. For example, *Y. pestis* expresses a form of lipopolysaccharide with reduced stimulation for the normal host receptor, TLR4 (Montminy et al. 2006). *Yersinia* LPS also provides resistance to the bactericidal effects of serum, in conjunction with the *Yersinia* proteins YadA and Ail. LcrV is another *Yersinia* protein capable of suppressing inflammatory responses by inducing production of the anti-inflammatory cytokine IL-10 (Brubaker 2003). As discussed above, *Yersinia* also translocates effector Yops into mammalian cells in order to block phagocytosis by innate immune cells. Moreover, YopJ inhibits cytokine production and can induce apoptosis in phagocytes. The importance of Yop-dependent resistance to innate immune recognition was recently demonstrated using coinfections in a mouse model of disease. Virulent *Y. pseudotuberculosis* induced profound inflammation in the gastrointestinal tract but survived, while coinfecting Yop-deficient bacteria were cleared (Logsdon and Mecsas 2006). Thus, *Yersinia* uses multiple strategies to evade innate immune recognition, replicate to higher levels, and cause more disease.

If innate immunity does succeed in suppressing *Yersinia* infection, the bacteria can also impair ensuing adaptive immune responses. YopJ indirectly inhibits T cell generation by directly affecting dendritic cell activation and function. YopO can induce apoptosis in T cells, and YopH can suppress T and B cell activation in culture. The ability of *Yersinia* to disable adaptive immune responses likely contributes to the pathogen's ability to cause disease and/or sequelae such as reactive arthritis.

However, the arsenal of *Yersinia* virulence mechanisms can be overcome if hosts are first made immune to *Yersinia*, an approach that is currently being used to understand the nature of successful anti-*Yersinia* immune responses and to develop effective vaccines. Several vaccines have been developed (see below). In addition to the potential for vaccines to protect humans and animals from *Yersinia* infection, these vaccines are extremely useful for dissecting the components and dynamics of protective immune responses to *Yersinia* in model hosts. Studies of vaccinated animals have

revealed that *Yersinia*-specific humoral responses are important (Titball and Williamson 2001). Antigens targeted by humoral immunity (i.e., antibodies) include the capsule Fraction 1 (F1) and V antigens, and likely function by allowing for opsonization of extracellular bacteria. A role for *Yersinia*-specific T cell responses has previously been downplayed due to the assumption that *Yersinia* are largely extracellular pathogens (Viboud and Bliska 2005), while T cells detect intracellular pathogens (Janeway et al. 2005). But there is abundant evidence to suggest that *Yersinia*-specific CD4+ and CD8+ T cells are required to protect against *Y. pestis* or *Y. enterocolitica* infection (e.g., see Hancock et al. 1986; Autenrieth et al. 1992; Parent et al. 2005; Philipovskiy and Smiley 2007). However, we know neither the identity of the *Yersinia* antigens recognized by T cells, nor how T cells restrict *Yersinia* infection. It has been suggested that IFN-γ secretion by T cells is important (Autenrieth et al. 1994), presumably by activating antimicrobial functions in neighboring phagocytes. Our understanding of effective anti-*Yersinia* immune responses will likely expand in the next several years due to increased interest in plague vaccination to prevent plague outbreaks that could result from bioterrorism.

VACCINES

Currently there is no licensed plague vaccine in the United States. In the past, both killed whole cell bacteria and live attenuated bacteria have been used in humans, but they are unsatisfactory for multiple reasons. Immunization with killed bacteria requires multiple doses, can cause side effects, and is of questionable efficacy, despite circumstantial evidence of protection against bubonic plague in immunized military personnel during service in the Vietnam War. Killed vaccines, however, do not protect against pneumonic plague and thus are of limited usefulness. Conversely, immunization with the live attenuated bacterial strain EV76 requires only a single dose and protects against both bubonic and pneumonic plague. Unfortunately, this strain, which lacks both the HPI and the *hms* locus, is only partially attenuated and can revert to full virulence, making it unsuitable for use in humans. More promising attenuated *Y. pestis* strains include those that lack YopH (Bubeck and Dube 2007) or enzymes important for outer membrane biogenesis (Feodorova et al. 2007). Another possible candidate may be *Yersinia* strains lacking components of the type III secretion system, as such strains lacking genes for the Yop secretion machinery (*ysc*) are attenuated and conferred protective immunity against virulent challenge via multiple routes, including intranasal.

The lack of a broad and effective bacterial vaccine has led to investigations of subunit vaccines. An acellular *Y. pestis* vaccine has been developed in the United Kingdom and is showing much potential in protecting against bubonic, systemic, and pneumonic forms of plague (Williamson et al. 2000). This vaccine is composed of two antigens, FI capsule and V antigen, and protects when delivered parenterally in emulsified adjuvant or intranasally in encapsulated microspheres (Prentice and Rahalison 2007). An alhydrogel-adjuvanted F1-V subunit vaccine is currently in phase II trials. Another subunit vaccine consists of orally administered attenuated *Salmonella typhi* expressing recombinant plague antigens, although this system is not as developed as the subunit vaccine (Calhoun and Kwon 2006). Regardless of delivery context, a cautious view of F1-V vaccine formations should be taken, given the potential for bioengineering of *Y. pestis*, the apparent rapid evolution of *Y. pestis*, and the observation that the FI antigen is not necessary for virulence. Nature or people could generate an organism that lacks the F1 antigen and does not express the immunoreactive epitopes in LcrV. Therefore, it seems prudent to continue to investigate this and other vaccines and therapeutics to use against possible future outbreaks of the deadly *Y. pestis.*

Vaccines against the enteric *Yersinia* are not a priority given the self-limiting nature of the infection in humans and the apparent commensality *Yersinia* has for many animal reservoirs. For yersiniosis in aquaculture, commercial bacterin vaccines (formalin-inactivated whole bacteria) have been used previously, but they are ineffective under severe stress conditions. Additionally, new immunization-resistant biogroups have emerged, underscoring the need for a new targeted vaccine against *Y. ruckeri* (Fernandez et al. 2007).

FUTURE DIRECTIONS

In the past 30 years, much progress has been made in understanding the proteins involved in the pathogenesis of *Yersinia* sp., particularly the pYV-encoded type III secretion system apparatus, Yops, iron acquisition mechanisms, and adherence factors

in the enteric *Yersinia* sp.; however, there remain areas that are still not completely understood. For instance, the adhesins that function in *Y. pestis* have not been completely identified or characterized; many of the genes that are critical for lung colonization by *Y. pestis* are unknown; and much remains to be learned about the genes responsible for the virulence of *Y. ruckeri*.

Another active area of research is identification of the genetic basis for the differences in clinical symptoms between the relatively mild enteric *Y. pseudotuberculosis* pathogen and the closely related, but much more virulent, *Y. pestis*. This line of investigation touches upon some ethically and politically sensitive areas of research, such as, "What are the genes needed to make a bacterium more virulent?" While addressing this question may provide researchers with the knowledge or means to generate a more lethal form of enteric *Yersinia* sp., not addressing this question seems ultimately foolish. An understanding of how pathogens establish footholds in hosts and negate host defenses will lead investigators to an understanding of how to interrupt the deadly life cycle, bolster host defenses against invading pathogens, and/or design therapeutics that target critical virulence factors. In the 1990s two independent cases of *Y. pestis* infection occurred in Madagascar in which the infecting bacteria had acquired genes encoding resistance to antibiotics. This acquisition of antibiotic-resistant genes presumably occurred in the flea (Hinnebusch et al. 2002). While antibiotics are not always effective against cases of pneumonic plague or systemic plague, they remain the most widely used and available treatment for *Y. pestis* infection. If *Y. pestis* becomes resistant to antibiotics, then the need for development of a *Y. pestis* vaccine or other therapeutics will become even more pressing.

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17
Pasteurella

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INTRODUCTION

Bacteria belonging to the genus *Pasteurella* are nonmotile, gram-negative organisms that are often found as normal oropharyngeal flora in many vertebrate species, but can also be primary or opportunistic pathogens. They are the causative agents of numerous diseases that are of significant economic importance to livestock industries including fowl cholera (FC) in birds, hemorrhagic septicemia (HS) in ungulates, and atrophic rhinitis (AR) in swine. They are also important pathogens in a range of zoonotic infections in humans. The genus is named in honor of Louis Pasteur who in the 1880s first recognized *Pasteurella multocida* as the causative agent of a fatal disease in chickens ("*choléra des poules*"—now termed FC) and showed that a laboratory attenuated *P. multocida* strain could be used as a live vaccine to stimulate protection against the disease.

The *Pasteurellae* are coccobacillary- to rod-shaped organisms measuring 0.3–1.0 µm in width and 1.0–2.0 µm in length. They can grow under aerobic, microaerophilic, or anaerobic conditions, and grow optimally between 37°C and 41°C. Most species in the genus are oxidase and catalase positive and can ferment glucose and other sugars with the production of acid but not gas. They can be cultured on rich media such as brain heart infusion and medium containing ruminant blood but not on MacConkey agar or Simmons citrate medium.

CHARACTERISTICS AND SOURCES OF THE ORGANISMS

In 1887 Trevisan proposed the genus name *Pasteurella* to describe bacteria associated with the diseases FC in birds and HS in cattle (Mutters et al. 1985). Initially a number of different species names were used, but the current name *P. multocida* was proposed in 1938 to include "typical, indole-producing, non-hemolytic hemorrhagic septicemia organisms" (Rosenbusch and Merchant 1939). The genus currently contains 12 named species (table 17.1) as given in the list of bacterial names with standing in nomenclature (www.bacterio.cict.fr/index.html; updated November 2007). However, phylogenetic analyses based on DNA–DNA hybridization (Mutters et al. 1985) and the similarity of 16S rRNA genes and various housekeeping genes (*infB*, *rpoB*, *sodA*, and *atpD*; Korczak and Kuhnert 2008) strongly suggest that only *P. multocida*, *Pasteurella dagmatis, Pasteurella canis*, and *Pasteurella stomatis* are members of *Pasteurella sensu stricto*.

Pasteurella multocida is the species most commonly associated with diseases that are of economic importance, with *P. multocida* subsp. *multocida* being the most commonly identified subspecies. It is the species that will be discussed in detail in this chapter. It is a heterogeneous species and a range of serological and genetic methods have been developed to allow differentiation of strains. Two serological methods are currently used to type *P.*

Table 17.1. Currently Recognized Taxa in the Genus *Pasteurella*, Host Predilection and Diseases

Species	Hosts	Association/diseases (common serotypes)
P. multocida subsp. *multocida*, *gallicida*, and *septica*	Birds, mammals	FC of birds (A, F, rarely D) Bovine pneumonia (A:3) AR of pigs (toxigenic serotypes A and D) HS of ungulates (B:2; B:2,5; E:2, E:2,5) Bite wound-associated infections in humans
P. dagmatis	Dogs	Normal flora in dogs. Cause a range of zoonotic infections in humans
P. canis	Cats and dogs Cattle and sheep?	Normal flora in cats and dogs. Cause a range of mostly bite wound-associated infections in humans. Pneumonia in cattle and sheep?
P. stomatis	Cats and dogs	Normal flora in cats and dogs. Cause a range of mostly bite wound-associated infections in humans
[P]. aerogenes[a]	Pigs	Sepsis, pneumonia, and diarrhea
[P]. bettyae[a]	Humans	Genitourinary infections
[P]. caballi[a]	Horses and pigs	Respiratory infections
[P]. langaaensis[a]	Birds	Normal flora of respiratory tract
[P]. pneumotropica[a]	Cats, dogs, and rodents	Pneumonia and various suppurative infections in rodents
[P]. mairii[a]	Pigs	Isolated from pig reproductive tract and associated with abortions
[P]. skyensis[a]	Fish	Fatal infections in Atlantic salmon
[P]. testudinis[a]	Tortoises	Respiratory disease in tortoises

[a] While these species are currently valid within the *Pasteurella* genus, genomic data suggest that they are not part of the *Pasteurella sensu stricto* group and that they will be moved to new genera in the future (Korczak and Kuhnert, 2008).

multocida strains. The first method, developed by Carter (1952), is a passive hemagglutination test that utilizes erythrocytes sensitized with capsular antigens and recognizes five serogroups (A, B, D, E, and F) based primarily on capsular polysaccharide. It is now clear from structural and genetic studies that strains from each of these types express distinct polysaccharide capsules (see below). The second serological method, developed by Heddleston et al. (1972), is a gel diffusion immunoprecipitation test that recognizes 16 serotypes based on expressed lipopolysaccharide (LPS) antigens. Recent structural studies have elucidated that each of the 16 Heddleston serotypes expresses structurally distinct LPS molecules (St. Michael et al. 2005a, 2005c; A. Cox, personal communication). The standard serotyping system for *P. multocida* encompasses both of these serological tests, with the capsule type stated first, followed by the LPS type (e.g., a designation of A:1 is capsular type A, Heddleston LPS type 1). Although this is the standard serotyping scheme, the methods are time-consuming and require access to the specific typing antisera. These antisera are difficult to generate, especially against the capsular polysaccharides, which are very poorly immunogenic. For this reason, a number of genetic typing methods have been developed in an effort to allow wider access to accurate and discriminatory typing methods (Blackall and Miflin 2000). Genetic typing methods also allow finer discrimination than that given by serological methods and may be useful for differentiating specific clonal isolates within and between outbreaks.

Polymerase chain reaction (PCR)-based assays have been designed to distinguish *P. multocida* from other bacterial species. The first described was based on primers specific for the gene *kmt1*, a pre-

dicted esterase/lipase (Townsend et al. 1998). The second PCR was based on primers to the 23S rRNA gene (Miflin and Blackall 2001). Both PCR assays were able to amplify a DNA product from all *P. multocida* strains tested, including strains from each of the *P. multocida* subspp. *multocida*, *gallicida*, and *septica*. Although the 23S rRNA assay was reported to give a false positive reaction with *Pasteurella avium* biovar 2 strains, and both assays were reported to give false positive reactions against *P. canis* biovar 2 strains, more recent genetic evidence suggests that these strains should be considered as authentic *P. multocida* strains (Christensen et al. 2004). Therefore, both of these assays appear to be *P. multocida*-specific.

A PCR-based assay has been designed to differentiate strains from each of the *P. multocida* capsular serotypes (A, B, D, E, and F; Townsend et al. 2001). This assay uses five primer pairs, each specific for sequences within the capsule biosynthetic locus of the different capsular serotypes, and is rapid and highly reliable (Davies et al. 2004). While it allows determination of the genetic capsule locus type and gives an identification even when classical Carter serotyping fails, it is not a phenotypic test and may give a positive result for strains that do not express a capsular polysaccharide as a result of mutation.

DISEASES CAUSED BY *PASTEURELLA MULTOCIDA*

Various strains of *P. multocida* are considered to be the primary causative agents of FC in birds, HS in ungulates, AR in pigs, and snuffles in rabbits. In addition, *P. multocida* can be a major contributor to some less well-defined conditions of the respiratory tract such as the Bovine Respiratory Disease Complex (BRDC), enzootic pneumonia of swine, enzootic pneumonia of calves, and pneumonia in sheep. The organism is commonly found in the oropharyngeal flora of dogs and cats and *Pasteurella* spp. are the most common organisms associated with cat and dog bite wound infections in humans.

FC is an important disease affecting both wild and domesticated birds worldwide. It manifests as epornitics of peracute to acute septicemias or chronic forms with localized infections of wattles, nasal sinuses, joints, ovaries, or other tissues. There is much overlap, and birds with chronic infections may recover, or develop the fulminating form of FC (Saif 2008). The disease causes significant economic losses to poultry industries throughout the world, particularly in turkeys (Carpenter et al. 1988). However, there are no recent figures for economic losses. In wild birds, FC is estimated to be the most important disease of North American wildfowls and significant outbreaks occur regularly in areas where waterfowls congregate in large numbers on wintering grounds, during migration, or in breeding colonies (Samuel et al. 2005). In large outbreaks more than 30,000 deaths have been recorded (Montgomery et al. 1979; Blanchong et al. 2006).

Hemorrhagic septicemia is a rapidly fatal systemic disease of ungulates that is most prevalent in Asia and Africa. Once clinical signs are observed, death is imminent. Most Asian countries rank HS as the most economically important bacterial disease of production and work animals (De Alwis 1999). Reported annual losses from the disease vary widely between countries; this variability is due not only to the differences in the size and importance of cattle, buffalo, and pig herds in each country but is also influenced by different surveillance and diagnostic capability. However, estimates of the disease being responsible for approximately 45% and 34% of all bovine deaths in India and Pakistan, respectively, and annual economic losses of US$350 million in Pakistan highlight the importance of the disease to these national economies (De Alwis 1999).

AR is a swine disease that is characterized by nasal turbinate destruction and maxillary dysplasia. Affected animals are more susceptible to chronic bronchopneumonias, from which other strains of *P. multocida*, usually capsular type A, may be recovered. The most severe form of the disease, known as progressive AR (PAR), results from infection with toxigenic strains of *P. multocida* (the strains that express the *P. multocida* toxin [PMT]). In pigs with PAR, coinfection of *P. multocida* and *Bordetella bronchiseptica* is often observed and *B. bronchiseptica* infection may facilitate colonization by *P. multocida* (Chanter and Rutter 1989). The disease results in economic losses to pig industries worldwide, since infected pigs show a 6–10% reduced daily weight gain (Donko et al. 2005).

"Snuffles" is subacute to chronic rhinitis affecting housed rabbits. Strains of capsular type A are most commonly involved, but capsular type D strains have been isolated. Rhinitis may be followed by, or associated with, bronchopneumonia, or with localized infections such as otitis media, pyometra, and arthritis. An AR-like syndrome of growing rabbits,

which is assumed to have a similar etiology and pathogenesis to the swine disease, has also been described (DiGiacomo et al. 1989).

In addition to the named syndromes, *P. multocida* may be associated with acute and chronic bronchopneumonias in virtually any species, but particularly in cattle, sheep, and swine. In most cases it is considered to be an opportunistic invader. The conditions that predispose to infection are poorly defined, but physiological stress, aspiration of heavy loads of particulate matter, and prior infection by other bacteria and viruses have been implicated.

PASTEURELLA MULTOCIDA: BACTERIAL VIRULENCE FACTORS

Many studies have focused on identifying *P. multocida* genes that are critical for virulence. As *P. multocida* can grow as a commensal organism, an opportunistic pathogen or a primary pathogen, the determinants of pathogenicity clearly involve not only bacterial products but host products and/or responses as well. Therefore, identified bacterial virulence factors can only be considered valid in the animal model in which they were tested. Indeed, there is now clear evidence that some *P. multocida* products are critical for virulence in some but not all hosts (e.g., lipopolysaccharide [LPS], which is more important in chickens than mice; Harper et al. 2003).

Capsule

Structure

Most *P. multocida* strains produce a polysaccharide capsule on their surface. Different *P. multocida* strains that express capsular polysaccharides with different chemical compositions have been identified. Carter type A strains express a polysaccharide capsule composed primarily of hyaluronic acid, a high molecular weight polymer with the repeating structure →4)-β-D-glucuronic acid-(1→3)-β-D-N-acetyl glucosamine-(1→ (Rosner et al. 1992). Carter type D strains express a polysaccharide capsule composed of unmodified heparin with the repeating structure →4)-β-D-glucuronic acid-(1→4)-α-D-N-acetyl glucosamine-(1→ (DeAngelis et al. 2002) while Carter type F strains express a capsule composed of unmodified chondroitin with the repeating structure →4)-β-D-glucuronic acid-(1→3)-β-D-N-acetyl galactosamine-(1→ (DeAngelis et al. 2002). These capsular polysaccharides are all glycosaminoglycans (GAGs) that are identical in primary structure to eukaryotic extracellular matrix components, although eukaryotic heparin and chondroitin are sulfated while the bacterial capsular polysaccharides are not. In eukaryotes, GAGs are essential structural components, and they play critical roles in cell-to-cell adhesion and in the regulation of the activity of various proteins. The presence of these molecules in the eukaryotic host means that there is no, or very limited, host recognition of bacteria encapsulated by these polysaccharides. This molecular mimicry is critical for the bacteria to avoid host immune responses.

The exact chemical structure of the polysaccharides expressed by Carter type B and E strains has not been elucidated but the type B molecule is composed of mannose, arabinose, and galactose. There are no published studies on the composition of the type E capsular polysaccharide.

Genetics of Capsule Biosynthesis

The nucleotide sequences of the capsule biosynthetic loci of *P. multocida* types A and B have been completely determined (Chung et al. 1998; Boyce et al. 2000) and the sequences of the genes involved in serotype-specific polysaccharide biosynthesis identified for types D, E, and F (Townsend et al. 2001; fig. 17.1). The genetic organization of each locus shows similarity to the group II capsule biosynthetic operons in other gram-negative bacteria and the genes can be grouped into three regions. Region 1 contains genes predicted to encode proteins involved in export of the polysaccharide to the surface via an ATP-binding cassette transport system; the genes in region 1 are highly conserved among all five serotypes. Involvement of *hexA* (serotype A:1) and *cexA* (serotype B:2) in transport of the polysaccharide to the bacterial surface has been confirmed by mutagenesis (Boyce and Adler 2000; Chung et al. 2001). Region 2 contains genes predicted to encode proteins involved in synthesis of the serotype-specific polysaccharides. Each of the synthases responsible for the polymerization of the hyaluronic acid (PmHAS), heparin (PmHS1), and chondroitin (PmCS) polysaccharides expressed by serotypes A, D, and F, respectively, has been confirmed by mutagenesis and/or functional assays (DeAngelis et al. 1998; DeAngelis and Padgett-McCue 2000; Kane et al. 2006). These synthases are bifunctional transferases, capable of addition of both the glucuronic acid and N-acetyl glucosamine

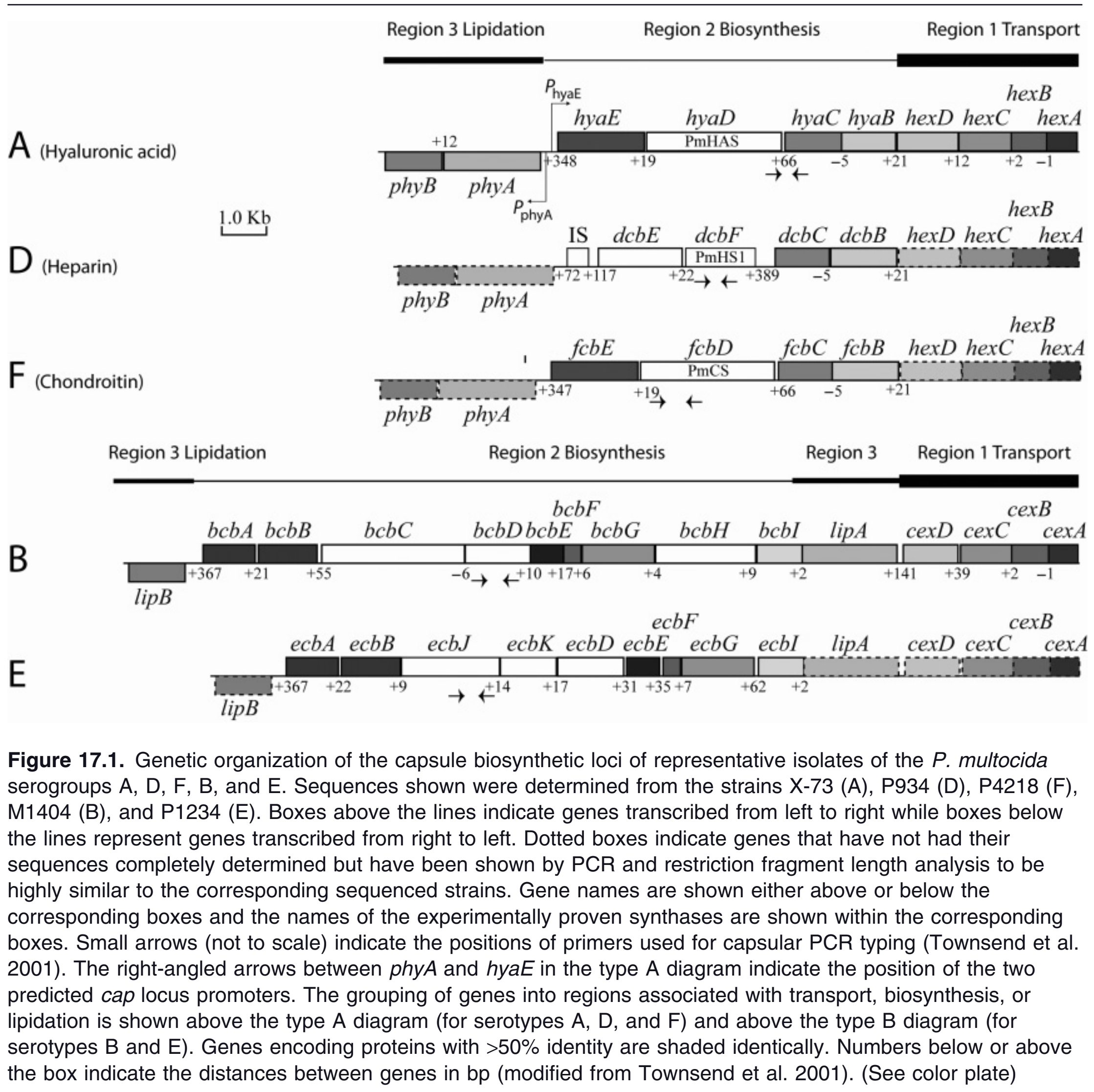

Figure 17.1. Genetic organization of the capsule biosynthetic loci of representative isolates of the *P. multocida* serogroups A, D, F, B, and E. Sequences shown were determined from the strains X-73 (A), P934 (D), P4218 (F), M1404 (B), and P1234 (E). Boxes above the lines indicate genes transcribed from left to right while boxes below the lines represent genes transcribed from right to left. Dotted boxes indicate genes that have not had their sequences completely determined but have been shown by PCR and restriction fragment length analysis to be highly similar to the corresponding sequenced strains. Gene names are shown either above or below the corresponding boxes and the names of the experimentally proven synthases are shown within the corresponding boxes. Small arrows (not to scale) indicate the positions of primers used for capsular PCR typing (Townsend et al. 2001). The right-angled arrows between *phyA* and *hyaE* in the type A diagram indicate the position of the two predicted *cap* locus promoters. The grouping of genes into regions associated with transport, biosynthesis, or lipidation is shown above the type A diagram (for serotypes A, D, and F) and above the type B diagram (for serotypes B and E). Genes encoding proteins with >50% identity are shaded identically. Numbers below or above the box indicate the distances between genes in bp (modified from Townsend et al. 2001). (See color plate)

or N-acetyl galactosamine to the molecule. Involvement of the product encoded by *bcbH* in synthesis of the type B polysaccharide has been confirmed by mutagenesis (Boyce and Adler 2001). Region 3 contains genes predicted to be involved in lipidation and surface attachment of the polysaccharides; these genes are highly conserved among all five serotypes.

The level of capsule expression is reduced in the presence of certain antibiotics and under conditions of reduced free iron (Jacques et al. 1991, 1994; Melnikow et al. 2008). However, there is no published information on the mechanism of capsule regulation. Analysis of a spontaneously arising acapsular strain indicated that it had highly reduced transcription across the *cap* locus (Watt et al. 2003). Although this initial report did not rule out the possibility that the reduced transcription was due to mutations within the *cap* promoter regions, we have recently shown that loss of transcription across the *cap* locus in similar spontaneously arising acapsular mutants is the result of mutations within a transacting transcriptional activator. We have identified this regulator as Fis and shown by RT-PCR and DNA

microarray analysis that Fis regulates both of the capsule promoters (between *phyA* and *hyaE*) in the type A locus (fig. 17.1) and at least 15 other genes/operons including *pfhB2*, which encodes a filamentous hemagglutinin.

Role in Virulence

Although many experiments have shown reduced virulence for spontaneous acapsular strains, or strains treated with capsule-degrading enzymes, the most definitive virulence studies have used isogenic capsulated and acapsular mutants. Defined acapsular mutants have been constructed in type A and type B strains and these mutants display highly reduced virulence in both chickens and mice (Boyce and Adler 2000, 2001; Chung et al. 2001). The type A mutant was highly susceptible to killing during growth in 90% avian serum with active complement. Furthermore, while the wild-type strain was able to grow rapidly *in vivo* in chicken muscle, the acapsular mutant was rapidly killed (60-fold reduction in 4 h). The acapsular type B strain was between four- and sixfold more sensitive to macrophage uptake *in vitro* and was rapidly cleared from the blood, liver, and spleen of infected mice to below detection limits after 4 h. Therefore, the presence of capsule is a critical barrier that allows *P. multocida* strains to avoid innate host immune defenses.

PMT

Pasteurella multocida toxin is a potent mitogen expressed by isolates belonging mainly to capsular type D and is responsible for AR in pigs (Foged et al. 1987; Lax and Chanter 1990). The pathological signs of disease include turbinate bone degeneration that results from the inhibition of osteoblast differentiation required for the regulation of bone-resorbing osteoclasts and bone formation. Other signs include the proliferation of both the regional turbinate epithelial cells and bladder epithelium cells (Lax and Chanter 1990; Hoskins et al. 1997; Harmey et al. 2004).

The PMT is a 146-kDa protein encoded on a lysogenic bacteriophage residing only in the genome of the toxin-producing strains of *P. multocida* (Pullinger et al. 2004). The toxin is passively released upon bacterial lysis and is believed to be actively taken up by host cells via receptor-mediated endocytosis by binding to a ganglioside cell surface receptor (Pettit et al. 1993). The N-terminal receptor-binding domain is then proteolytically cleaved under low pH conditions (Smyth et al. 1995), exposing the translocation domain, which allows uptake into the cytoplasm (Baldwin et al. 2004). Here the catalytic C-terminal portion of the protein activates two heterotrimeric G proteins, $G\alpha_q$ and $G\alpha_{12/13}$, which in turn affect a number of host signal transduction pathways (Lax et al. 2004). Activation of $G\alpha_q$ in the phospholipase Cβ signal transduction pathway results in a number of cellular effects, including the constitutive activation of signal transducers and activators of transcription (STAT) proteins 1, 3, and 5 (Orth et al. 2007); activation of these proteins is oncogenic in some systems. Activation of $G\alpha_{12/13}$ by PMT stimulates the Rho-linked signaling pathway, resulting in major cytoskeletal changes, inhibition of osteoblast differentiation, and activation of the mitogen-activating protein (MAP) kinases (Lacerda et al. 1996; Harmey et al. 2004).

In vitro, PMT has been shown to elicit maturation of human dendritic cells by activation of the phospholipase C signaling pathway, but interestingly does not appear to act as a mucosal adjuvant, actively blocking migration of dendritic cells to regional lymph nodes (Bagley et al. 2005; Blocker et al. 2006).

Iron-regulated and Iron-acquisition Proteins

Almost all bacteria require iron for survival. The genome of *P. multocida* strain Pm70 encodes 53 proteins with homology to known iron metabolism or acquisition proteins (May et al. 2001). *P. multocida* has a number of mechanisms to sequester iron from precipitated ferric hydroxides via siderophores or from the iron-rich host proteins such as heme and transferrin. In all cases, uptake of iron requires a specific outer membrane receptor and a periplasmic-binding protein. Energy for all iron uptake systems is supplied by the TonB complex consisting of the proteins TonB, ExbB, and ExbD anchored to the inner membrane (Krewulak and Vogel 2008). Given the critical role this complex plays in iron uptake, it is perhaps not surprising that *tonB*, *exbB*, and *exbD* mutants of *P. multocida* are fully attenuated (Fuller et al. 2000; Bosch et al. 2002a).

In other gram-negative bacteria, iron is extracted from transferrin while bound to an outer membrane complex consisting of a TbpA dimer associated with a surface exposed lipoprotein TbpB, predicted to play a role in the initial binding of transferrin (Boulton et al. 1999). Transfer of the sequestered

iron from the outer membrane to the cytoplasm occurs via the FbpA periplasmic-binding protein (reviewed in Krewulak and Vogel 2008). Interestingly, there is no TbpB homolog identified in *P. multocida*, and the TbpA receptor is unique in its ability to bind transferrin without TbpB (Ogunnariwo et al. 1991; Ogunnariwo and Schryvers 2001). Based on PCR analysis, the TbpA gene was present in most ruminant *P. multocida* isolates, but not in isolates from other hosts (Ewers et al. 2006). There have been no other transferrin receptors identified in *P. multocida*, but this does not preclude the possibility that TbpA^{-} strains can utilize transferrin using an unidentified receptor. Indeed, a microarray study of the avian *P. multocida* Pm70 strain revealed up-regulation of many iron-related genes in response to transferrin as the only iron source (Paustian et al. 2002), showing that this strain responds to the presence of transferrin.

For bacteria to access the iron within hemoglobin, appropriate enzymes are required to first lyse host red blood cells. Under normal aerobic growth *P. multocida* has no clear hemolytic activity, but the bacteria have been shown to lyse erythrocytes under anaerobic conditions. The enzyme responsible for this activity has not been identified; although it has been demonstrated that a *P. multocida* esterase, MesA, can indirectly confer hemolytic activity on *Escherichia coli* (Hunt et al. 2000).

Eight *P. multocida* outer membrane receptors that bind either hemin or hemoglobin, or both, have been identified (Bosch et al. 2002b 2004; Cox et al. 2003). Six of these, including HgbA, HgbB, and HbpA, were shown to bind both hemoglobin and hemin, while HemR bound only hemoglobin, and Pm1282 bound only hemin (Bosch et al. 2004). Two independent studies in different *P. multocida* strains demonstrated that inactivation of *hgbB* does not alter the virulence of *P. multocida* in mice (Cox et al. 2003; Bosch et al. 2004). However, there are conflicting reports on the role of HgbA in virulence. A signature-tagged mutagenesis study using a serotype A bovine isolate identified a transposon mutant of *hgbA* displaying significant reduction in *in vivo* growth and virulence in CD-1 mice (Fuller et al. 2000). However, in another study a defined *hgbA* mutant of a serotype D:12 ovine *P. multocida* isolate was shown to be fully virulent in female Swiss mice and bound hemoglobin *in vitro* at wild-type levels (Bosch et al. 2002b). Clearly, most *P. multocida* strains express multiple receptors for iron uptake from hemoglobin, giving rise to redundant uptake systems.

Siderophores are structurally diverse, small, iron-chelating compounds expressed by many microorganisms. These molecules can be classified into three structural types: hydroxycarboxylate, catecholate, or hydroxamate. Only one siderophore, named multocidin, has been studied in *P. multocida*, and although its structural type was not identified, chemical tests determined that it was not a catechol- or hydroxamate-type siderophore (Hu et al. 1986). Three outer membrane proteins were shown to bind to ferric-bound multocidin under iron-limiting conditions, but the specific receptor for multocidin was not identified (Choi-Kim et al. 1991).

Early research demonstrated an increase in outer membrane protein (OMP) expression from *P. multocida* cells grown in iron-depleted medium, and immunization with these OMPs conferred better cross-protective immunity than immunization with OMPs derived from cells grown in standard media (Glisson et al. 1993; Ruffolo et al. 1998). However, while DNA microarray analyses comparing *P. multocida* gene expression during growth in iron-rich and iron-depleted medium identified a large range of genes with altered expression; proteomic studies have revealed relatively few OMPs with altered expression in iron-depleted medium (Paustian et al. 2001, 2002; Boyce et al. 2006).

LPS

Structure

LPS is an important structural component of the gram-negative outer membrane. Enteric gram-negative bacteria express LPS that has a variable number of polymeric O-antigen repeats, but *P. multocida*, like other pathogens such as *Haemophilus influenzae*, *Haemophilus ducreyi*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae*, expresses LPS that lacks a repeating O-antigen.

The structure of the *P. multocida* LPS isolated from the serotype A:1 strain VP161 has been examined in detail. This strain expresses equal amounts of two different LPS glycoforms designated A and B. These glycoforms share an identical outer core structure but their inner core structures differ (St. Michael et al. 2005b). Notably, the inner core of glycoform A has a single phosphorylated Kdo residue whereas that of glycoform B contains two un-phosphorylated Kdo residues (Harper

et al. 2007a). The simultaneous expression of two different inner core structures attached to a conserved oligosaccharide extension is unusual and has only been observed in *P. multocida* and the closely related species *Mannheimia haemolytica*. A third LPS glycoform, designated glycoform C, is expressed at low levels by a VP161 *hptE* mutant (Boyce et al. 2009) and some strains from other Heddleston serotypes. It has the novel terminal tetrasaccharide β-galactose-(1-4)- β-N-acetyl glucosamine-(1-3)- β-galactose-(1-3)- β- N-acetyl glucosamine-(1-4). These terminal sugars mimic residues present on eukaryotic glycosphingolipids. Interestingly, other pathogens such as *H. influenzae* and *N. meningitidis* express LPS molecules with a similar terminal extension. It is possible that simultaneous expression of these multiple LPS glycoforms confers some *in vivo* survival advantage to *P. multocida*.

Genetics of LPS Biosynthesis

Bioinformatics analyses and directed mutagenesis of the *P. multocida* strain VP161 have allowed the identification of the genes encoding each of the transferases essential for the synthesis of LPS glycoforms A and B. Although the genes responsible for the synthesis of the inner core section of the LPS molecule (*hptA*, *hptB*, *hptC*, *hptD*, *gctA*, *gctB*, *kdkA*) are scattered around the genome, genes encoding the transferases required for the synthesis of the outer core section are located together between the conserved and non-LPS biosynthesis related genes *priA* and *fpg*. This outer core LPS locus is highly variable, consistent with the diversity of outer core LPS structures in the 16 different Heddleston serotypes.

Role of LPS in Virulence

The importance of LPS in the pathogenesis of FC has been studied extensively using the highly virulent *P. multocida* A:1 strain VP161 (Harper et al. 2004 2007b; Boyce et al. 2009). Any mutation that reduces the length of the expressed LPS also reduces virulence in the FC disease model. Indeed, loss of just the terminal phosphocholine residues caused a significant reduction in virulence. Interestingly, outer core LPS mutants were highly attenuated in the chicken FC model, but were still able to cause disease in the mouse model.

Strains expressing truncated LPS were more susceptible to the chicken antimicrobial peptide fowlicidin. Loss of any of the outer core sugars increased susceptibility to fowlicidin, but strains lacking the inner core tri-heptose side chain were especially susceptible (Harper et al. 2004, 2007b; Boyce et al. 2009). It is presently unclear whether the increased susceptibility to host antimicrobial peptides such as fowlicidin is the sole reason for the attenuation of strains expressing altered LPS.

P. multocida LPS is endotoxic, but its toxicity varies between hosts. LPS isolated from serotype B strains and administered intravenously into buffalo can mimic the clinical signs of hemorrhagic septicemia (Horadagoda et al. 2002), but turkey poults were relatively resistant to the effects of LPS isolated from a serotype A strain (Ganfield et al. 1976; Rhoades and Rimler 1987; Mendes et al. 1994). In addition to different host responses, differences in the LPS structure in different serotypes may affect the nature and intensity of the innate immune response.

Adhesins and Outer Membrane Proteins

Colonization of host tissues is an essential process for infection to be established and bacteria express a range of proteins that enable initial binding to host cells. The *P. multocida* Pm70 genome encodes a range of putative fimbriae and adhesin proteins, including fimbrial genes *ptfA*, *flp1*, and *flp2*, and the filamentous hemagglutinin genes *hsf_1* and *hsf_2* (May et al. 2001). Type IV fimbriae, assembled using subunits of PtfA, have been identified on the surface of a number of *P. multocida* strains but their role in virulence has not been determined (Ruffolo et al. 1997). However, the Flp pilin subunits, the associated tight adherence protein TadD, and both filamentous hemagglutinin proteins PfhB1 and PfhB2 have all been identified as having a role in virulence (Fuller et al. 2000; Harper et al. 2003; Tatum et al. 2005). In adhesion studies, the outer membrane protein OmpA was shown to bind to Madin Darby bovine kidney cells and also to interact with the extracellular matrix components fibronectin and heparin, suggesting a role for this protein in binding to host cells (Dabo et al. 2003). Recent studies using phage display libraries have detected an additional adhesin, ComE1, that binds both fibronectin and double-stranded DNA, thereby combining two possible functions, namely adhesion to the host via fibronectin and natural transformation through binding of DNA (Mullen et al. 2008a, 2008b).

Sialometabolism

In almost all eukaryotes, sialic acid is an abundant constituent of cells and has a number of roles, including stabilization of membranes and regulation of transmembrane receptor function. Sialic acid is the only 9-carbon keto sugar found in prokaryotes and invading bacteria can utilize host sialic acid as a carbon and nitrogen source or as a source of amino sugars (Vimr et al. 2004). In addition, sialic acid can be synthesized *de novo* or obtained from the host for incorporation into cell surface components such as capsule or LPS. The addition of sialic acid to these surface polysaccharides may allow circulating bacteria to avoid and/or alter the host immune system and may promote biofilm formation (Vimr and Lichtensteiger 2002; Swords et al. 2004). *P. multocida* has a complete sialometabolic system comprising pathways for uptake, activation, transfer, and dissimilation of sialic acid but lacks the two enzymes, NeuB and NeuC, required for *de novo* biosynthesis of sialic acid from UDP-N-acetylglucosamine (Steenbergen et al. 2005). Instead, sialic acid is obtained from the host using the sialidases NanH and NanB, which have different specificities. NanH can utilize sialoglycoconjugates with either 2,3 or 2,6 sialated lactose; whereas NanB can only efficiently cleave 2,6 sialyl lactose (Mizan et al. 2000). It is not clear whether either sialidase is capable of cleaving sialic acid from endogenous LPS.

Current knowledge of *P. multocida* sialometabolism indicates that while it plays a nutritional role in the acquisition of carbon, nitrogen, and amino acids, its most critical role *in vivo* is likely to be the decoration of the bacterial cell surface. *P. multocida* encodes two sialyltransferases capable of transferring activated sialic acid to cell surface components. The best characterized of these is Pm0188, a unique multifunctional enzyme for which the crystal structure of the protein complexed with both donor and acceptor molecules has been determined (Ni et al. 2007). The major function of this enzyme is as an α2,3-sialyltransferase, transferring activated sialic acid to galactosides, but it can also act less efficiently as an α2,6-sialyltransferase at pH 4.5 to 7.0, as an α2,3 sialidase at pH 5.0–5.5, and as a *trans*-sialidase able to transfer α2,3 linked sialic acid from one galactoside to another (Yu et al. 2005). There are no published data on the specificity of the second sialyltransferase, Pm0508, but it is encoded within the same locus as the glycosyltransferases Pm0509, Pm0511, and Pm0512, which are all predicted to be required for the assembly of the terminal trisaccharide in the *P. multocida* VP161 glycoform C LPS (Boyce et al. 2009). Thus, the genomic location of *pm0508* strongly suggests a role in the sialic acid decoration of glycoform C LPS.

The presence of two sialyltransferases suggests that at least some *P. multocida* LPS glycoforms are sialated and recent experiments measuring sialic acid uptake in *P. multocida* strain Pm70 indicated that sialic acid is indeed incorporated into a cell surface component resembling LPS (Steenbergen et al. 2005). However, there are no published reports of sialic acid on any specific LPS structures, and we have been unable to detect sialated LPS in our structural studies on the LPS expressed by *P. multocida* strain VP161. However, inactivation of the sialyltransferase encoded by *pm0188* results in a significant attenuation of *in vivo* growth of *P. multocida* in chickens (our unpublished data), suggesting that this sialyltransferase is critical for virulence. The role of the second sialyltransferase (Pm0508) in virulence is less clear, as a *pm0508* mutant was significantly attenuated for growth both *in vitro* and *in vivo* (our unpublished data).

Inactivation of a number of the *P. multocida* sialometobolism genes (*nanE*, *nanA*, and *pm1709*) suggested that sialic acid uptake was essential for virulence, as inactivation of the gene encoding the predicted sialic acid-binding protein Pm1709 dramatically reduced *in vivo* fitness (Fuller et al. 2000). However, it is currently unclear whether sialic acid catabolism is important for growth *in vivo*, since inactivation of *nanA* (encoding an aldolase) did not affect *P. multocida* virulence in mice, while inactivation of *nanE* (encoding an epimerase) resulted in significant attenuation (Fuller et al. 2000; Steenbergen et al. 2005).

PATHOGENESIS

Overview

Numerous *P. multocida* virulence factors have been identified, but specific contribution to pathogenesis for many of them remains obscure. As *P. multocida* can cause diseases with a wide range of presentations in numerous hosts, there are likely to be significant differences between the pathogenesis of each disease. The variety of diseases and hosts may be due to any or all of the following: strain adaptation to hosts, variation in possession and/or

expression of specific virulence factors, host factors such as ecology, anatomic features, and innate immunity.

There is some consistency of association of *P. multocida* serotypes with specific hosts and disease syndromes; FC is generally caused by capsular types A and F, AR by D strains, and HS by type B and E strains (table 17.1). For some strains host specificity is very strong; for example, type B strains seem to be restricted to mammals, and in attempted experimental infections we have been unable to induce any disease in chickens by intramuscular injection of highly virulent serotype B:2 strains, even at doses of 10^6 CFU. However, many strains seem to be capable of forming at least temporary commensal relationships with a variety of species. For example, apparently healthy birds and mammals that were in contact with turkey flocks that experienced outbreaks of FC harbored strains of *P. multocida*, some of which were genotypically identical to the strains recovered from diseased birds (Snipes et al. 1989). However, it remains unclear how important cross-species contamination is to disease transmission and maintenance, and at present there are no data on the molecular or cellular basis of host specificity for diseases.

Interactions between Bacteria and Host Defenses

Apparently healthy animals may maintain oropharyngeal populations of *P. multocida* for long periods (De Alwis et al. 1990; Muhairwa et al. 2000), but the precise mechanisms by which bacteria can invade and colonize pulmonary or other tissue are not known. *P. multocida* does not normally cross healthy epithelium or the mucosa of the gastrointestinal tract of birds, yet it readily colonizes the lower respiratory tract if inoculated into the trachea and passes rapidly into the vascular system through the lower respiratory tract (Wilkie et al. 2000). Colonization of mammalian lung tissue usually requires predisposing factors such as a preceding virus infection (cattle) or *Mycoplasma* infection (pigs). It is likely that a range of stresses can cause alterations in previously healthy carriers that allow tissue invasion, resulting in local or systemic infection. Most strains of *P. multocida* that live as commensals are relatively innocuous to a healthy, immunologically competent host, yet virtually any strain has the potential to cause serious disease such as pneumonia or even fulminating, multisystemic infections under conditions of poor hygiene and overcrowding (Cameron et al. 1996).

For HS the natural route of infection appears to be via tonsillar tissue (De Alwis et al. 1990), whereas for FC and bovine respiratory disease complex (BRDC) the respiratory tract appears to be the primary portal. Infection of birds via the lower respiratory tract is easy to achieve with relatively small doses of bacteria and may be facilitated by macrophages (Wilkie et al. 2000). Once *P. multocida* enters the tissue or blood, it may multiply locally (wound infection, HS, pneumonia) or localize temporarily in organs such as the liver and the spleen (birds) from where it may later spill out into the blood.

In the tissue, even low-virulence strains of *P. multocida* may grow profusely, causing marked local necrosis and a rapidly spreading cellulitis, particularly in the immunocompromised host (Gowda and Stout 2002; Tattevin et al. 2005). Whether or not *P. multocida* persists within the host and moves beyond local infection depends on both the bacterial strain and the host response. Bacterial capsule and LPS play a critical role by conferring resistance to different components of the host's innate immune system (Boyce and Adler, 2000; Chung et al. 2001; Harper et al. 2004). There is some correlation between the expressed polysaccharides and the host specificity of various strains (table 17.1). A strong inflammatory response is usually invoked when *P. multocida* grows in any parenchymatous tissue, but in immunologically naïve animals this may not be effective in preventing systemic spread of infection.

Fulminating diseases are often referred to as "septicemias" (FC, HS), but definitive evidence for bacterial multiplication in blood *in vivo* is lacking. In fact, bacterial numbers may remain very low in blood while multiplying rapidly in tissue sites such as the liver in FC (Pabs-Garnon and Soltys 1971). Indeed, although many strains can grow rapidly *in vitro* in serum with active complement, this does not necessarily correlate with virulence (Diallo and Frost 2000). Therefore, it is possible that the terminal bacteremias observed are due primarily to spillover from multiplication in tissue sites such as the liver (FC; Pabs-Garnon and Soltys 1971) or peri-tonsillar tissue (HS; De Alwis et al. 1990). Animals that die of fulminating disease typically have widespread petechiae on serosal and epicardial surfaces, an indication of the consumptive coagulopathy common to many endotoxemias.

Although the clinical signs and lesions of acute, fulminating disease can be largely attributed to endotoxin (LPS; Horadagoda et al. 2001 2002), and LPS contributes to bacterial survival *in vivo* (Harper et al. 2007a 2007b; Boyce et al. 2009), the precise role of LPS in early disease onset remains unknown.

We have recently shown that circulating chicken leukocytes produce different cytokine profiles in response to infection with a virulent (VP161) or an avirulent strain (VP17) of *P. multocida* (Jayaweera and Wilkie, unpublished results). Which of the structural or secreted factors expressed by the virulent strain modulates this effect is currently unknown. However, we have found that the avirulent strain VP17 expresses atypical LPS and has a mutation in the *hptE* gene (unpublished results). In VP161, HptE is essential for the expression of the major LPS glycoforms A and B but not for the expression of glycoform C (Boyce et al. 2009). Whether this LPS mutation in VP17 is responsible for the altered cytokine responses is the subject of ongoing studies.

Genomics and Genomic-Scale Studies in Understanding Pathogenesis

The advent of genome analysis, combined with DNA microarray transcription profiling and proteomic analysis, should lead to considerable advances in understanding the complexity of the pathogen-host interaction. Some examples of these approaches are described below. There is currently only one published annotated genome sequence available for the genus *Pasteurella*, namely the *P. multocida* strain Pm70 (May et al. 2001). The Pm70 strain was recovered from a case of FC in chickens and although initially reported as type A:3, analysis of its capsule biosynthetic locus suggests that it is actually capsular type F, as it encodes a predicted chondroitin synthase. A second *P. multocida* genome sequence, of a non-toxigenic serotype A isolate that was recovered from a pneumonic lesion in a pig, has recently been completed and although the sequence data are available (www.micro-gen.ouhsc.edu/p_mult/p_mult_home.htm) there is no published annotation.

The Pm70 genome comprises a single circular chromosome of 2,257,487 bp with an overall G + C% of 41%. The chromosome contains 2015 predicted protein-coding sequences and six rRNA operons. Analysis of the genome allowed prediction of 104 putative virulence-related genes, including two genes (designated *pfhB1* and *pfhB2*) encoding homologs of the *Bordetella pertussis* filamentous hemagglutinin, which is involved in the adhesion of the bacteria to host cells and is a major component of the human whooping cough vaccine. Inactivation of the *pfhB1* and *pfhB2* genes in *P. multocida* significantly reduced virulence (Fuller et al. 2000; Tatum et al. 2005). Analysis of the genome also indicated that *P. multocida* Pm70 contains 53 genes (2.5% of the genome) associated with iron metabolism and acquisition (May et al. 2001).

Genomic-Scale Transcriptional Profiling. DNA microarrays can measure the transcriptional response of every gene in an organism to various stimuli. Thus, these analyses offer the promise of elucidating how bacteria regulate their gene expression in response to the conditions encountered within some environmental niche (such as the eukaryotic host) and may allow the identification of genes critical for survival at those sites.

During growth inside a host, bacteria must survive and replicate in an immunologically hostile and nutrient-restricted environment that includes a low concentration of iron. The response of *P. multocida* to both low iron (Paustian et al. 2001), and a range of defined iron sources including transferrin, ferritin, and ferric citrate, has been explored (Paustian et al. 2001 2002). During the first 2 h of growth within medium with reduced iron, *P. multocida* showed altered expression of at least 135 genes. Many genes encoding proteins involved in iron transport were up-regulated, including *yfeABCD*, *fpbABC*, *fecBCD*, *tonB*, *exbBD*, *hgbA* (*pm0300*), *pm0336, pm0741, pm0803, pm1078,* and *pm1079* (Paustian et al. 2001). The proteins ExbB, ExbD, and TonB play a critical role in energy transduction for high affinity iron uptake systems and *P. multocida exbB*, *exbD*, and *tonB* mutants were all highly attenuated for virulence (Bosch et al. 2002a). While a *P. multocida* type A strain *hgbA* mutant was attenuated for virulence (Fuller et al. 2000), a type D strain *hgbA* mutant was not (Bosch et al. 2002b). A *P. multocida* type A strain *pm0803* mutant retained virulence (our unpublished data). Thus, although the proteins encoded by these genes are clearly important in iron uptake, they are dispensable for virulence at least in some strains, probably reflecting redundancy in these pathways. Other gene sets differentially regulated in response to low iron included genes involved

in energy production (*gapdh*, *pgk* and *eno* down-regulated and *lldD* up-regulated) and amino acid biosynthesis (*aroA*, *trpG*, *hisH* and *ilvM* all up-regulated).

The transcriptional response of *P. multocida* to growth in chicken liver and blood has been determined by whole genome microarrays (Boyce et al. 2002, 2004). In each case the gene expression pattern of the bacteria growing in BHI at 41°C was compared with the gene expression pattern of bacteria recovered from each host niche at the terminal stages of infection. During growth in blood, certain genes involved in amino acid transport and biosynthesis and nitrogen assimilation were highly up-regulated (*asnA*, *aspC*, *dppA*, *gdhA*, and *ilvH*). GdhA catalyzes the conversion of ammonia to glutamate and is a component of the primary pathway for the assimilation of nitrogen and the synthesis of amino acids. AspC is involved in the conversion of glutamate to aspartate, while AsnA catalyzes the production of asparagine from aspartate and ammonia. Thus, these enzymes are all involved in the mobilization of nitrogen from ammonia to amino acids, which suggests that the *in vivo* environment, at least at the terminal stages of infection, is ammonia-rich and amino acid-poor. Statistical analysis showed that the transcriptional response of bacteria recovered from the livers of chickens with FC was highly similar to that observed for bacteria recovered from blood (Boyce et al. 2004). Similar up-regulation of *gdhA*, *asnA*, and *dppA* was observed, but bacteria purified from liver showed a more marked up-regulation of genes involved in carbohydrate transport and metabolism and altered regulation of genes known to respond to anaerobic conditions. Interestingly, a gene expression pattern indicative of a switch to growth under anaerobic conditions was also observed for *P. multocida* grown *in vitro* with hemoglobin as the defined iron source (Paustian et al. 2002).

The transcriptional response of a *P. multocida* serotype A isolate to treatment with various antibiotics was assessed using DNA microarrays (Melnikow et al. 2008). Treatment with various bacteriostatic antibiotics altered the expression of between 18% and 40% of the *P. multocida* genes while treatment with several bactericidal antibiotics altered the expression of only a very few genes (0.4–1.3% of the genome; Melnikow et al. 2008). Importantly the bacteriostatic antibiotics (with the exception of rifampin) generally down-regulated expression of a range of predicted or known virulence genes including genes involved in capsule biosynthesis and iron uptake. It is currently unclear to what extent antibiotic-induced changes in virulence gene expression may contribute to bacterial clearance *in vivo* during antibiotic treatment of an infected host.

Proteomic Analyses. Recent analyses using combined two-dimensional gel electrophoresis (2-DGE) followed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) or one-dimensional gel electrophoresis followed by liquid chromatography tandem mass spectrometry (1-D LC MS/MS) have allowed the identification of specific *P. multocida* proteins in certain subcellular locations and shown how these proteins respond to environmental conditions.

The outer membrane is the site of direct interaction between bacteria and host, and many important virulence factors are expressed on the bacterial surface. Proteomic analysis of the *P. multocida* outer membrane proteome during growth *in vitro* in rich medium and in iron-depleted medium and *in vivo* in chickens with late stage FC identified approximately 30 proteins likely to be components of the outer membrane (Boyce et al. 2006). Expression of two of these outer membrane proteins (Pm0803, OmpW) was altered in response to both reduced iron and growth *in vivo*. OmpW expression was reduced during growth *in vivo* and in a medium with reduced iron, whereas Pm0803 expression was highly up-regulated during growth *in vivo* and in iron-depleted conditions. It is likely that the expression of both of these proteins is regulated specifically by the low iron concentration of each of these growth conditions. Pm0803 is a predicted outer membrane porin involved in iron uptake. Inactivation of Pm0803 did not affect virulence (our unpublished data), indicating that *P. multocida* has redundant systems involved in iron acquisition

Lesions

The lesions of the recognized syndromes for which *P. multocida* is responsible, or contributes significantly to, are well described in standard textbooks such as Jubb, Kennedy, and Palmer's "Pathology of Domestic Animals" (HS, AR) (Maxie 2007), or "Diseases of Poultry" (FC) (Saif 2008). In general, lesions are typical of those invoked by gram-negative pyogenic bacteria (pneumonia and

cellulitis) in acute and subacute conditions. In peracute to acute fulminating systemic infections such as HS and FC, the principal lesions are usually those associated with toxemias, such as widespread petechiae and evidence of cardiovascular collapse.

IMMUNITY IN *PASTEURELLA MULTOCIDA* INFECTIONS

Virtually all of the available information relating to immunity in pasteurellosis refers to infections with *P. multocida*. However, because of the broad host range of *P. multocida* and the diversity of animal models used to study immune responses and immunity, it is difficult to draw overall conclusions about the mechanisms of immunity. Indeed immunity will clearly be different according to the animal species and the type of infection. It is therefore not possible to extrapolate data obtained from infection with a specific serogroup, serotype, or strain in a particular animal species to other *P. multocida* infections.

Innate Immunity

Innate host defense mechanisms such as phagocytosis, the production of antimicrobial peptides, and complement are key components of nonspecific natural resistance to infection. However, *P. multocida* has evolved mechanisms for circumventing these in the naïve host. Bacterial capsules protect bacteria from phagocytosis and from the bactericidal action of complement. The capsule of *P. multocida* is critical in mediating evasion of phagocytosis by macrophages (Boyce and Adler 2000). A genetically defined mutant showed enhanced engulfment, thereby confirming earlier work that showed that *P. multocida* was in general resistant to phagocytosis by both macrophages and heterophils unless opsonized (Harmon et al. 1992) and that susceptibility to phagocytosis could be increased by enzymatic removal of capsule with hyaluronidase (Harmon et al. 1991).

The ability of *P. multocida* to survive and grow in the presence of fresh serum was abrogated by mutation of the capsule biosynthesis locus (Chung et al. 2001), consistent with an earlier work that showed a correlation between capsule and serum resistance (Hansen and Hirsh 1989). However, some very low-virulence strains can grow and multiply in 90% chicken serum (Diallo and Frost 2000); clearly other factors are also important for serum resistance.

Antimicrobial peptides produced by the host appear to play at least some role in innate immunity to FC-causing strains of *P. multocida* in chickens; there is no information for other serotypes. Wild-type *P. multocida* was resistant to the avian cathelicidin peptide fowlicidin, with resistance heavily dependent on expression of entire, full length LPS (Harper et al. 2007a 2007b). Fowlicidin is a cationic peptide and the charge-based interaction of the peptide with the negatively charged phosphate residues on lipid A is predicted to be an essential initial step in the antimicrobial action (Daugelavicius et al. 2000). *P. multocida* strains that express LPS lacking the terminal phosphocholine residues are significantly more susceptible to the action of fowlicidin (Harper et al. 2007b). It is likely that the charge on the phosphocholine residues helps to reduce the interaction of the cationic peptide with lipid A. However, strains that express LPS lacking the heptose side chain are much more susceptible to killing by fowlicidin, suggesting that these residues form a steric barrier to the peptide (Boyce et al. 2009).

Early work showing major histocompatibility complex (MHC)-linked resistance to FC in chickens has not been investigated further (Lamont et al. 1987).

Acquired Immunity

There is long-standing evidence that immunity to *Pasteurella* infection is mediated principally by antibody. Passive immunity can be transferred with immune serum from mice, rabbits, chickens, turkeys, and cattle. Additionally, immune serum has opsonic and bactericidal activity (Wijewardana et al. 1990; Ramdani and Adler 1991) and anti-LPS monoclonal antibodies are also opsonic and protective in mice (Wijewardana et al. 1990). Nevertheless, in chickens at least, cellular immunity also appears to be important; protective immunity could be transferred by immune spleen cells or by culture supernatant from immune splenocytes (Baba 1984). Immunity in bovine HS and bovine respiratory pasteurellosis is also presumed to be mainly antibody-mediated, as immune bovine serum can passively protect mice. However, the actual role of antibody in bovine immunity is far from clear, since some studies have shown a correlation of antibody with protection while others have found no relationship (Dabo et al. 2007). More work is therefore required to define the respective roles of humoral and cellular immunity in most species.

Attempts to manipulate the immune response to *P. multocida* have included the administration of melatonin to rats; immune modulation was dependant on the time of day of administration (Korde et al. 2005). Enhancement of both antibody response and protective immunity to *P. multocida* infection in mice was achieved by DNA-based delivery of a hybrid Interleukin-2 gene (Xie et al. 2007).

FC was one of the first bacterial infections against which vaccination was attempted. Louis Pasteur famously showed that repeated *in vitro* passage of *P. multocida* resulted in attenuation of the bacteria and that chickens inoculated with the attenuated strain were then resistant to challenge with a virulent strain. Of course, in Pasteur's strain the basis for attenuation was not known, with reversion to virulence a major potential problem. Nonetheless, this important work demonstrated the feasibility of immunoprophylaxis. Since then a range of vaccines has been investigated, mostly for use in cattle and buffalo or poultry. Why then, over 125 years later, do we still not have available an ideal vaccine? Currently each of the available vaccines has significant drawbacks. In general, bacterins give only short-term, serotype-specific immunity, with sometimes variable efficacy, and they must be delivered by injection. Conversely, live attenuated vaccine strains can be administered in food or water, and give better cross-serotype protection, but the currently available undefined, attenuated, strains have caused disease under some circumstances. While much effort has been focused on the development of a cross-protective subunit vaccine, there are none in current production. As well as vaccine efficacy, cost of vaccine production and administration is a critical consideration in vaccine development, as profit margins for growers are generally very small.

Bacterins and Cell Components

There is general consensus that bacterin vaccines can stimulate immunity in poultry and in cattle, but that immunity is relatively short-lived and is restricted to homologous or closely related serotypes. This does not constitute a major problem for HS, where the causative serotypes (B:2 and E:5) are closely related. Consequently, bacterins against HS have been used with relatively good success rates for over 100 years (De Alwis 1992; Verma and Jaiswal 1998); these vaccines are administered with a range of adjuvants usually consisting of oil-water emulsions. The effectiveness of these oil-adjuvant vaccines has been questioned from time to time and has been evaluated many times, but even a relatively recent study demonstrated efficacy in buffalo calves, although the numbers of animals were small (Shah et al. 2001). Mice vaccinated with a serotype B:2,5 vaccine were not protected against challenge with a B:3,4 strain. Vaccination against pneumonic pasteurellosis presents additional problems (reviewed by Dabo et al. 2007), with one study finding no protection in calves vaccinated intratracheally with killed bacteria (Dowling et al. 2004).

Cross-protective immunity is relevant in the development of poultry vaccines, where it is clear that laboratory-cultured, killed bacterial cells elicit only homologous protection. The reason for this restriction is almost certainly that important cross-protective protein antigens are poorly expressed by *P. multocida* during some conditions of *in vitro* growth. Bacteria harvested from infected tissues and then killed are able to stimulate heterologous immunity (Rebers and Heddleston 1977), which can be transferred by serum to naïve turkeys (Rebers et al. 1975). There is evidence that the cross-protective antigens may be expressed *in vitro* under low-iron conditions (Glisson et al. 1993; Ruffolo et al. 1998). The identity of some of the individual proteins is discussed below.

Live Vaccines

Empirically derived, live, attenuated strains of *P. multocida* have been used as vaccines against FC mainly in turkeys. The original Clemson University (CU) A:3,4 strain stimulates both humoral and cellular immunity and induces protection when administered in drinking water to turkeys, but not in chickens. Several derivatives of the CU strain have been developed, including temperature sensitive M-9, PM#1, and PM#3 strains unable to grow at 42°C *in vitro*. However, the mechanism of attenuation for all of these strains is unknown and the vaccines retain a degree of virulence under experimental conditions (Hopkins and Olson 1997) and have occasionally caused disease outbreaks in the field (Hopkins et al. 1998). Live vaccines for HS have been derived from field isolates, such as the serotype B:3,4 strain isolated from deer (Myint et al. 1987). Again, while demonstrating a good degree of efficacy, the basis for the observed attenuation is unknown and these vaccines have not gained widespread usage (Verma and Jaiswal 1998).

Genetically defined, attenuated strains capable of stimulating protective immunity have been constructed. Mutation of the *aroA* gene leads to attenuation of *P. multocida* due to the inability of the bacteria to synthesize aromatic amino acids, whose low concentrations in host tissues results in reduced growth *in vivo*. The defined *aroA* mutants of A:1 and A:3 FC-causing strains stimulate heterologous protection in both mice (Homchampa et al. 1992 1997) and in chickens (Scott et al. 1999). Similar attenuated *aroA* mutants have also been constructed in a B:2 background strain. Systemic or intranasal vaccination of mice elicited solid immunity (Tabatabaei et al. 2002). Unfortunately, in calves intramuscular injection of the vaccine was necessary to stimulate protective immunity, with intranasally vaccinated calves remaining completely susceptible (Hodgson et al. 2005; Dagleish et al. 2007).

Genetically defined acapsular mutants are attenuated in both A:1 and B:2 backgrounds (Boyce and Adler 2001; Chung et al. 2005), but their potential as vaccines has not been developed further.

Individual Antigens

The identity of protective antigens in *P. multocida* infections has remained surprisingly elusive. It is clear that LPS plays some role in immunity; anti-LPS monoclonal antibodies are opsonic and can transfer at least partial protection, but immunisation of mice with LPS failed to elicit any protection (Wijewardana et al. 1990; Ramdani and Adler 1991). LPS-based immunity is by definition restricted to the homologous serotype.

A cross-protective protein antigen of 39-kDa purified from *P. multocida* grown *in vivo* (Rimler 2001) was localized to the bacterial cell surface using a monoclonal antibody, but not identified (Rimler and Brogden 2001). However, the protective antigen was later identified erroneously as the lipoprotein PlpB (Tabatabai and Zehr 2004), which shows similarity to methionine-binding proteins from many other bacterial species. In fact, the cross-protective antigen is PlpE, a lipoprotein with homologs in *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae* that stimulate protective immunity in cattle and pigs (Wu et al. 2007). Recombinant soluble PlpE elicited cross-protection in mice and chickens; significantly, immunization with PlpB resulted in no protection in either mice or chickens (Wu et al. 2007). Work in the authors' laboratory has confirmed these findings and also shown that insoluble PlpE is able to stimulate protective immunity in chickens and mice.

The major outer membrane porin OmpH is likewise able to stimulate protective immunity. Anti-OmpH monoclonal antibodies protected mice via opsonization (Vasfi Marandi et al. 1996). Work in chickens also showed that OmpH was a protective antigen. Interestingly, native but not recombinant OmpH was protective, as was a cyclic peptide corresponding to a predicted exposed loop of the protein (Luo et al. 1999); other peptides were not active. A subsequent study claimed protection of mice with recombinant fragments of OmpH (Lee et al. 2007), although statistical significance was marginal. No chicken experiments were performed. Recently, a mutant in the regulatory gene *fur* was used to generate outer membranes rich in iron-regulated proteins (including OmpH); vaccination with outer membranes purified from this mutant stimulated immunity in mice (Garrido et al. 2008), although the numbers of animals were small, thus making statistical analysis difficult. Intriguingly, the absence of OmpH in a second mutant increased the degree of cross-protection conferred.

Other vaccine studies with individual proteins that showed some level of protection included a 37.5-kDa (Lu et al. 1991) and a 39-kDa protein (Ali et al. 2004; Sthitmatee et al. 2008). However, in these studies the identity of these proteins was not determined and it is very likely that these proteins were actually PlpE. No protection was seen following immunization of mice with OmpA or turkeys with P6 protein. No vaccines based on individual protein antigens have been developed commercially.

Immunity against porcine AR is dependent on antibodies against the dermonecrotic toxin, PMT, elaborated by AR-causing strains of *P. multocida*; disease can be reproduced experimentally with purified toxin. Accordingly, a range of toxin derivatives has been developed for successful immunization against AR. Approaches have included formaldehyde-inactivated toxin (Foged et al. 1989), the construction of non-toxigenic derivatives by specific site-directed mutagenesis (S1164A and C1165S; To et al. 2005), and the generation of recombinant toxin fragments (Liao et al. 2006).

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Despite more than 125 years of research, *Pasteurella* remains an enigmatic pathogen. Although a large range of mammalian and avian species can be infected experimentally, in natural infection *Pasteurella* spp. exhibit a degree of host specificity, or at least host predilection, which appears to be related to the nature of the capsule expressed on the bacterial surface. However, the molecular and cellular basis of this specificity remains completely unknown. For example, why are serogroup B strains exquisitely virulent for bovine and murine hosts but not for chickens? The precise mechanism of bacterial transmission from one host to another remains likewise obscure and is likely to involve respiratory and digestive tracts as well as traumatic breaches of the integument. Much still remains to be learned about the site of initial colonization that precedes systemic spread and how *Pasteurella* crosses mucosal surfaces to gain access to the bloodstream and other tissues. A probable reason for this lack of knowledge has been the fact that tools for the genetic analysis and manipulation of *Pasteurella* have lagged behind those that have been available for many other bacterial species for more than 25 years.

However, recent developments in genomics, proteomics, and molecular biology have the potential to facilitate major advances in understanding, in the next few years, the pathogenesis of *P. multocida* and other *Pasteurella* infections. The availability of genome sequences and the readiness with which genomes of multiple strains can now be sequenced easily and cheaply will allow comparative genomics analyses to shed considerable light on the variable virulence phenotypes and host specificity displayed by *Pasteurella*. Likewise, the ability to construct defined mutants in genes of interest will define the roles of many genes and gene products in the causation of disease by *Pasteurella* and in the role of the host response in both pathogenesis and immunity to infection.

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18
Mannheimia

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INTRODUCTION

The genus *Mannheimia* was introduced in 1993 to include a number of microorganisms that previously belonged to the *Pasteurellaceae* family. Prior to the introduction of *Mannheimia*, members of the *Pasteurellaceae* consisted of *Haemophilus*, *Actinobacillus*, and *Pasteurella* (Pohl 1981). Within the genus *Pasteurella*, there were several species with the major pathogens being *Pasteurella haemolytica* and *Pasteurella multocida*. *P. haemolytica* was subdivided into two biotypes and 17 serotypes. Biotypes A and T were based on the ability to ferment L-arabinose or trehalose, respectively. The various serotypes were established based on surface soluble or extractable polysaccharide molecules in hemagglutination tests (Biberstein et al. 1960). The serotypes 1, 2, 5–9, 11, and 12 belonged to biotype A whereas serotypes 3, 4, 5, and 15 belonged to biotype T. However, using DNA–DNA hybridization data as well as rDNA sequence analysis, it was shown that the two biotypes only had DNA homology between 3% and 13%, indicating that they were distinct microorganisms. Further, it was also shown based on DNA homology that *P. haemolytica* and *P. multocida* were very different and should not be placed in the same genus. In 1999, all *P. haemolytica* biotype A serotypes 1, 2, 5, 6, 7, 8, 9, 12, 13, 14, 15, and 16 were reclassified in a new genus *Mannheimia* and were named *M. haemolytica* (Angen et al. 1999). The *P. haemolytica* serotypes 3, 4, 10 (belonging to biotype T) were reclassified as *Pasteurella trehalosi*. *P. haemolytica* serotype 11 was placed in a separate genus as *Mannheimia glucosida*.

CHARACTERISTICS AND SOURCES OF *MANNHEIMIA*

Since the establishment of the *Mannheimia* spp., the following microorganisms have been classified in this genus: *M. haemolytica*—isolated from cattle and sheep, this includes all *P. haemolytica* A biotypes; *M. glucosida*—isolated from sheep, previously *P. haemolytica* serotype 11; *Mannheimia granulomatis*—isolated from cattle; *Mannheimia ruminalis*—isolated from sheep rumen; *Mannheimia succiniciproducens*—isolated from the bovine rumen, discussed below; and *Mannheimia varigena*—isolated from cattle. Each of these classifications was based primarily on DNA–DNA hybridization and/or 16s rDNA sequence comparison.

Most studies of *M. haemolytica* have been performed on pathogenic isolates due to their association with bovine pneumonic pasteurellosis. Healthy animals are nasopharyngeal carriers of *M. haemolytica* belonging to different serotypes but the relative abundance changes during an infection since usually only serotypes A1 and A6 are recovered from pneumonic lungs. *M. haemolytica* A1 is the principal cause of bovine pneumonic pasteurellosis, also known as shipping fever, a fibrinopurulent infection and sometimes necrotizing bronchopneumonia in cattle. Serotype A1 has been the predominant microorganism isolated from cattle that die of this type of acute respiratory disease.

Recently, however, there has been an increase in the isolation of *M. haemolytica* serotype A6 from diseased animals, suggesting a change in the infection rates caused by the different serotypes (Donachie

2000), perhaps the result of immunization of cattle using vaccines produced with serotype 1-based antigens. Most commercially available vaccines are prepared from serotype 1 cultures and/or extracts from serotype 1 bacterial cells. Hence, cattle may be protected from infection by serotype 1 microorganisms, but become susceptible to infection by strains of another serotype such as serotype 6. It would be of interest to compare the genetic and particularly the antigenic differences between serotypes 1 and 6 to identify unique features of serotype 6 that may allow it to be a successful pathogen in the presence of immunization against serotype 1.

Mannheimia succinoproducens is a gram-negative bacterium isolated from bovine rumen. It was initially placed in the genus *Mannheimia* based on greater than 94% identity in its 16s rDNA sequence comparison with the other *Mannheimia* spp. It is a capnophilic bacterium with a high tolerance of low O_2 and an affinity for CO_2. It is involved in the fermentation process in the rumen using fumarate as the electron acceptor with the production of volatile fatty acids (such as succinic acid) in the anaerobic environment. There is no evidence that it is pathogenic. The genome sequence of *M. succinoproducens* strain MBEL55E has been determined (Hong et al. 2004) and found to consist of a circular DNA of 2.3 Mbp with a mol% G + C of 42.5. The initial genome comparison based on 16S rDNA sequence suggested that it was closely related to *P. multocida* Pm70. A recent analysis using highly conserved housekeeping genes suggested a separation between *M. succinoproducens* and *M. haemolytica* and a reevaluation of the relationship between these species. Even though these two bacteria were both isolated from cattle, they occupy different niches and have likely diverged significantly.

BACTERIAL VIRULENCE FACTORS

This section is limited to discussion of *M. haemolytica*. The genes that code for a number of its virulence factors have been isolated and characterized (Highlander 2001; Lo 2001). These include a secreted leukotoxin, a sialoglycoprotease, outer membrane proteins, transferrin-binding proteins, adhesins, and genes for biosynthesis of capsule, and lipopolysaccharide (LPS). The characterization of these virulence genes allows researchers to determine the likely role of each factor in pathogenesis. In addition, some of these virulence factors have been used as candidates in the development of vaccines to protect cattle from bovine pneumonic pasteurellosis.

Leukotoxin (LktA)

The leukotoxin is the major secreted protein that plays a major role in the pathogenesis of *M. haemolytica*. It was originally observed that the bacterium produces a heat-labile molecule that has a cytolytic activity specific against bovine leukocytes (Markham and Wilkie 1980; Shewen and Wilkie 1982). This molecule was present in the cell-free culture supernatant of the bacterium. Subsequently, the genes that code for the leukotoxin were cloned and sequenced (Lo et al. 1985; Highlander et al. 1989). Analysis and comparison of the deduced amino acid sequence of the leukotoxin with information from the data banks showed that the *M. haemolytica* leukotoxin (LktA) is highly homologous to the α-haemolysin of *Escherichia coli* (Strathdee and Lo 1987). Understanding of the α-haemolysin guided the design of experimental approaches. For example, the toxin structural gene *lktA* codes for the LktA of 102 kDa and was found to be activated by LktC by an acylation process (Bauer and Welch 1996). The mechanism of secretion of LktA was found to be similar to that of the α-haemolysin. Subsequently, the genes that code for the secretion proteins LktB and LktD were located immediately downstream of *lktC* and *lktA* (Strathdee and Lo 1989a). The expression of the *lkt* operon was also found to be similar to that of the α-haemolysin (Highlander et al. 1990). A promoter upstream of *lktC* directs the synthesis of two different mRNA molecules. A short mRNA codes for *lktC* and *lktA*; whereas a longer mRNA codes for all four genes in the operon (Strathdee and Lo 1989b). The mechanism that determines the production of the full-length mRNA and the shorter mRNA is not known but it most likely involves some type of transcription termination signal at the end of *lktA*. There have been a number of reports on several loci and/or mechanisms that influence expression of the *lkt* operon (Highlander et al. 1993), but there is still no information on the system or systems that regulate the expression of the *lkt* operon.

The leukotoxin acts by insertion into the target cells and by formation of a pore of approximately 0.9 nm in diameter (Clinkenbeard et al. 1989). This was concluded to result in leakage of ions from the cells leading to osmotic imbalance and cell lysis. Recently, however, this mode of action of the

leukotoxin was challenged since exposure of bovine lymphocytes to low doses of leukotoxin resulted in DNA fragmentation, a characteristic of apoptosis (Sun et al. 1999). Atapattu and Czuprynski (2005) also reported that the leukotoxin induces apoptosis of bovine lymphoblastoid cells (BL-3) by a caspase-9-dependent pathway. Subsequently, they showed that leukotoxin bound to lymphocyte function-associated antigen 1 (LFA-1) on BL-3 cells and was located in lipid rafts and clathrin-coated pits via a dynamic process that resulted in its internalization and in cytotoxicity (Atapattu and Czuprynski 2007). Inhibition of clathrin coat production reduced leukotoxin internalization and leukotoxin-mediated cytotoxicity to BL-3 cells. Furthermore, inhibition of actin polymerization reduced both leukotoxin internalization and leukotoxin-mediated cytotoxicity. The authors suggested that both lipid rafts and clathrin-mediated mechanisms are important for leukotoxin internalization and cytotoxicity in BL-3 cells.

More recently, Aulik and Czuprynski (2008) showed that, after entry into BL-3 cells, leukotoxin binds to the mitochondria, resulting in impairment of mitochondrial function and contributing to death of neutrophils. These additional activities of the leukotoxin at various doses lead to the final outcome of loss of function of the neutrophils or cell lysis, which is one of the contributing factors to the pathology of the pneumonic lungs. The importance of the leukotoxin to pathogenesis has been reinforced by Highlander et al. (2000) who created an *lktC* mutant that is incapable of activating the leukotoxin. The results showed that this mutant was less virulent than the parental strain.

The mechanism of target cell specificity of the leukotoxin has also been addressed. Leukotoxin specifically binds to β2 integrins on the target cells (Li et al. 1999; Jeyaseelan et al. 2000) and only recognizes and interacts with integrins from bovine sources (Deshpande et al. 2002). Additional experiments based on these studies showed that strains that have specificity against other animal species produce leukotoxins that recognize the integrins from those hosts (Dassanayake et al. 2007).

The leukotoxin from serotype A1 is highly active against leukocytes from bighorn sheep, stressing the importance of leukotoxin in pneumonia in bighorn sheep (Dassanayake et al. 2008, 2009).

Transferrin-Binding Protein

In order to survive and multiply in the mammalian host, the invading bacterium has to secure iron. Iron availability in a mammalian host is limited as the host has evolved mechanisms to sequester the iron from invading pathogens. It has been long suspected that *M. haemolytica* must have specific iron-acquisition systems for it to be a successful pathogen. *Mannheimia haemolytica* produces iron-binding proteins that exhibit specificity for bovine transferrin (Ogunnariwo and Schryvers 1990). Interestingly, all isolates tested were incapable of using human, porcine, avian, or equine transferrin, demonstrating species specificity in its iron usage. Subsequently, the researchers showed that these strains produced an iron-regulated protein of 100 kDa that was involved in the binding of bovine transferrin. Two other proteins of 77 and 71 kDa were also identified in these studies. The *tbpB, tbpA* genes that coded for the transferrin-binding receptors TbpA and TbpB have been cloned and sequenced (Ogunnariwo et al. 1997). Nucleotide sequence analysis of the genes showed that they are likely encoded in an operon arrangement of *tbpB-tbpA*. The availability of recombinant TbpB allowed researchers to demonstrate the specificity of TbpB for ruminant transferrin. Subsequently, studies were carried out with recombinant TbpA and TbpB in a vaccine trial and challenge experiment in cattle. Animals vaccinated with TbpA and TbpB together showed a high resistance to challenge and the level of anti-TbpB antibodies was correlated to resistance (Potter et al. 1999).

LPS and Capsule

These two outer cell surface components are important to virulence and/or survival of *M. haemolytica* cells in the host. However, both molecules are synthesized from multiple components and there is no single protein that serves as the antigenic unit.

The LPS of most gram-negative microorganisms is an important part of the outer membrane and often plays a role in virulence. The typical LPS is divided into three components: the lipid A, the core, and the O-antigen. The lipid A moiety is involved in the anchoring of the LPS into the outer membrane. It consists of phospholipids and lipids. The lipid A unit, known as endotoxin, has the toxic and pyrogenic effect of LPS. Lipid A is often associated with anaphylactic response if it is present in vaccine components. The core oligosaccharide can be

divided into inner core and outer core. It usually consists of KDO moieties, which are the sites for the attachment of the O-antigen. The O-antigen consists of various lengths of repeating sugar moieties of the same or multiple sugars. It has been suggested that the O-antigen units protect the bacterial cell from complement as the membrane attack complex is formed away from the membrane and cannot exert its action. The composition of the LPS core of serotype A1 consists of D-glucose, D-glycero-D-manno-haptose, and L-glycero-D-manno-heptose (Lacroix et al. 1993). There is also evidence of a D-galactose as the terminal nonreducing core moiety. A number of genes involved in LPS biosynthesis have been characterized in *M. haemolytica* (Potter and Lo 1995, 1996). Whether there is a direct role for LPS during pathogenesis has not been clearly demonstrated. However, it is likely that uncontrolled proliferation of the bacteria in the lungs, in the absence of phagocytic cells because of LktA killing, would result in LPS-associated inflammation. On the other hand, there have been reports that the LPS is associated with the leukotoxin and may contribute to leukotoxin activity and/or stability (Li and Clinkenbeard 1999). The partial efficacy of rough mutant *E. coli* vaccines in protection against bovine acute pneumonic disease argues for the importance of LPS in pathogenesis.

The capsule of *M. haemolytica* has been implicated in a role of survival in the host (Brogden et al. 1989; Chae et al. 1990) and also shown to modulate the antibacterial activities of bovine neutrophils (Czuprynski et al. 1989). The capsule of serotype A1 is composed of N-acetyl-D-mannosanuronic acid linked 1–4 to N-acetyl-D-glucosamine (Adlam et al. 1984). The degree of encapsulation of *in vitro*-grown cultures has been shown to be growth phase-dependent. Cells from log phase cultures appear to have higher encapsulation (Gentry et al. 1982). A comparison of the chemical composition of the capsule from serotype A1 with other bacterial carbohydrates showed that the capsule of A1 is similar to the enterobacterial common antigen (ECA). This similarity was subsequently supported by analysis of the capsule biosynthetic locus (see below).

The genetic cluster for capsule biosynthesis has been cloned and sequenced (Lo et al. 2001). The locus can be divided into three regions similar to that of the typical group II capsule biosynthetic clusters in gram-negative bacteria. Region 1 contains four genes that code for an ATP-binding cassette transport apparatus for the secretion of the capsule materials across the membranes. Two of the *M. haemolytica* A1 genes, *wzt* and *wzm*, complemented the *E. coli kpsT* and *kpsM* mutants, respectively. The ATP-binding activity of Wzt was demonstrated by its affinity for ATP agarose, and the lipoprotein nature of Wza was supported by [^{3}H]-palmitate labeling. Region 2 contains six genes; four genes (orf1/2/3/4) code for unique functions with no identifiable homologs. The remaining two genes (*nmaA* and *nmaB*) encode homologs of UDP-N-acetylglucosamine-2-epimerase and UDP-N-acetylmannosamine dehydrogenase, respectively. These two proteins are highly homologous to the *E. coli* WecB and WecC proteins that are involved in the biosynthesis of ECA. Complementation of an *E. coli wecB/C* mutant with the *M. haemolytica* A1 *nmaA/B* genes resulted in the restoration of ECA biosynthesis. Region 3 contains two genes (*wbrA*, *wbrB*) that are suggested to be involved in the phospholipid modification of capsular materials. An acapsular mutant has been made by the inactivation of the *nmaA* and *nmaB* genes (McKerral and Lo 2002). Serum sensitivity assays indicated that the acapsular mutant was as resistant as the encapsulated parent to complement-mediated killing by colostrum-deprived calf serum but was more sensitive to killing by immune bovine serum. A comparison of LPS prepared from the acapsular mutant and encapsulated parent confirmed that these strains have long O-polysaccharide chains, possibly conferring resistance to serum-mediated killing. However, the experiment using the acapsular mutant to examine the virulence of the mutant in an infection in cattle has not been done.

Outer Membrane Proteins

A number of outer membrane proteins from *M. haemolytica* have been characterized (Craven et al. 1991). A recombinant plasmid that codes for three outer membrane lipoproteins, Plp1/2/3, was first reported by Cooney and Lo in 1993. The three lipoproteins were homologous to a *Haemophilus influenzae* 28-kDa lipoprotein. Plp-1,-2, and -3 were found to be antigenic components in a culture supernatant vaccine, hence they could be candidates for further investigation as vaccine components. A fourth lipoprotein was subsequently characterized (Nardini et al. 1998). This lipoprotein Plp4 is homologous to a 31-kDa antigen from *Histophilus somni* and a 19.2-kDa antigen from *Neisseria meningitidis*. Additional homology of the distal half of

Plp4 was identified with a number of bacterial outer membrane proteins belonging to the OmpA family. Plp4 appears to be a novel type of outer membrane protein that contains motifs typical of OmpA but that is also lipid modified.

Using an immunological screen, Lo and Mellors (1996) isolated a collection of clones each coding for a different secreted antigen of *M. haemolytica*. From this collection, they characterized the Gs60 antigen in detail.

When the complete sequence of the gene that coded for the GS60 antigen was compared with the data banks, it was found that a partial fragment of the gene had been previously identified (Weldon et al. 1994). In that study, the researchers screened a fusion library of *M. haemolytica* and identified an antigen of approximately 6 kDa. Their study showed that a high antibody titer to this antigen was correlated with disease resistance. Subsequently, the complete gene and its full length sequence were found by Lo and Mellors (1996). Analysis of the GS60 antigen showed that it belongs to the LppC family of bacterial outer membrane lipoproteins (Marchler-Bauer et al. 2005). The N-terminal of the protein contains a bacterial lipobox consensus sequence consistent with the cysteine residue being lipid modified. The function of these bacterial LppC proteins is unknown. Additional studies showed that serum from calves vaccinated with a commercial vaccine and demonstrating protection against *M. haemolytica* contained antibodies against GS60 (Lee et al. 2008). Additional data also showed that GS60 is immunogenic and *in vivo* expressed in calves, consistent with the suggestion that GS60 plays an important role during an infection (Lo et al. 2006). A derivative of GS60 has been expressed in transgenic alfalfa in the development of an edible vaccine (see below).

O-Sialoglycoprotease

Otulakowski et al. (1983) observed that the culture supernatant of *M. haemolytica* contains an enzymatic activity that cleaves *O*-sialoglycoproteins. Subsequently, this activity was shown by Abdullah et al. (1992) to be due to a neutral metalloprotease with specificity for *O*-sialoglycoprotein. The 35-kDa enzyme cleaves human erythrocyte glycophorin A, which is *O*-glycosylated, but does not cleave *N*-glycosylated proteins or non-glycosylated proteins. Glycophorin A was cleaved when it was present *in situ* in erythrocyte ghost plasma membranes or when it was free in solution. Activity of the glycoprotease is inhibited by EDTA, citrate, and ascorbate, but inhibition appears to be due to the masking of metal ion activators rather than to their removal. The gene that codes for this *O*-sialoglycoprotease has been cloned and sequenced (Abdullah et al. 1991). Additional analysis showed that the *gcp* gene is present in all A biotypes of *P. haemolytica* (Abdullah et al. 1990). Subsequently, it was shown that calf serum contains antibodies against *O*-sialoglycoprotease (Lee et al. 1994) and that this sialoglyprotease may have IgG1 protease activity (Lee and Shewen 1996). Finally, a recombinant form of Gcp was used in a vaccine combination and showed protective activity in a vaccine trial study (Shewen et al. 2003), suggesting that this antigen should also be included in vaccine preparations.

Adherence and Colonization

After bacteria have been inhaled into the alveoli, they must overcome the mechanisms present that remove such foreign particles. The continuous exhalation of air should remove bacteria not firmly attached to the cell surfaces. In addition, the presence of mucus and ciliary movement also reduces the ability of the bacterium to remain in the host tissue environment. To persist in the host's alveoli, *M. haemolytica* is likely to have mechanisms that facilitate its attachment and colonization in the host. Fimbria-like structures have been observed by electron microscopy on the cell surface of *P. haemolytica* (Morck et al. 1987, 1988). However, there is no further information on these structures. Potter et al. (1987) reported purification of a 12-nm fimbria with a molecular mass of 35 kDa from *M. haemolytica*.

Jaramillo et al. (2000) described the purification of an adhesin protein from *M. haemolytica* using immobolized GlcNAc and NeuAc and suggested that these molecules are the binding receptors on the host cells. The purified 68-kDa adhesin was named MhA (De la Mora et al. 2006) and the receptor subsequently shown to be a 165-kDa glycoprotein on neutrophils (De la Mora et al. 2007).

We recently observed the ability of *M. haemolytica* to bind to fibronectin (Lo and Sorensen 2007). Fibronectin is one of the major extracellular matrix proteins found on the surface of eukaryotic cells. Gram-positive and gram-negative bacterial binding to fibronectin has been well documented (Signas

et al. 1989; Sperandio et al. 1995). The bacterial proteins involved in fibronectin binding have been well characterized (Hansky and Caparon 1992; Menzies 2003). We showed that *M. haemolytica* binds to fibronectin in a dose-dependent manner. Furthermore, this binding ability is due to a trypsin-sensitive molecule on the cell surface. By using immobilized fibronectin as the bait, we purified a 35-kDa outer membrane protein from *M. haemolytica* that binds to fibronectin. Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry identified it as outer membrane protein A (OmpA), a protein commonly found on the outer membranes of most gram-negative microorganisms, that has binding activities mapped to a series of four loops that protrude from the membrane. Davies has previously examined OmpA proteins from several *Pasteurellaceae* microorganisms and predicted that the loops must be involved in a specific interaction between the microorganisms and their host (Davies and Lee 2004). OmpA has also been examined in detail by Smith who named it a molecular Swiss army knife (Smith et al. 2007). They compared various OmpA proteins from *E. coli* and showed that the four major loops of OmpA are involved in invasion of brain microvascular endothelial cells (BMEC). In particular, loops 1 and 2 have been proposed to interact with gp96, a glycoprotein on BMEC cells. Therefore, it appears that *M. haemolytica* is using OmpA to facilitate attachment and colonization.

Trimeric Adhesin

Trimeric adhesins are autotransporter proteins that secrete themselves without accessory components (Henderson et al. 2004). To date, all trimeric autotransporters have been shown to have adhesive functions (Cotter et al. 2005). These trimeric adhesins are characterized by lollipop-like structures displayed on the cell surface formed by the stalk and head domains of the proteins (Kim et al. 2006). Each of the monomer unit consists of approximately 70 amino acid residues that can form four β-strands at the C-terminal end. Three of these units together produce a 12-stranded β-barrel unit for translocation of the adhesin through the outer membrane, hence the name trimeric autotransporter. A crystal structure of the Hia adhesion from *H. influenzae* was solved by Meng et al. (2006). The structure showed the 12-stranded β-barrel structure had a central channel of 1.8 nm in diameter. There is also a conserved neck region that connects the adhesin head to the stalk region (Linke et al. 2006). The stalk region consists of a series of coiled-coiled arrangements that support the adhesin domain toward the receptor on the host cell.

We have recently identified an *ahs* locus in *M. haemolytica* that codes for a trimeric adhesin (Daigneault and Lo 2008). The *ahs* locus codes for two proteins, AhsA and AhsB, which makes it a member of the two-partner secretion system for the secretion of large proteins (Jacob-Dubuisson et al. 2001). AhsA is a 70-kDa protein that contains multiple collagen-binding domains. There are 20 collagen-binding domains found in two clusters of 9 and 11 domains. The motifs are found in most cases in a 14-amino acid repeat. In addition, two conserved "neck" sequences with high similarity to the neck sequences in Yad were also present in AhsA. The neck sequences in the *Yersinia enterocolitica* adhesin (YadA) are involved in surface localization of the adhesion domain, stalk formation, and oligomerization of the adhesin on the cell surface (Roggenkamp et al. 2003). AhsB is the cognate partner of AhsA and is most likely involved in secretion of the adhesin. AhsB contains a 46% amino acid similarity with other trimeric adhesins at the C-terminal end of the protein. This C-terminal region has been shown to form a β-barrel structure for translocation of the passenger protein. In particular the last six amino acids of AhsB are identical to the characteristic pattern present in other autotransporter translocator domains.

Collagen-binding experiments showed that *M. haemolytica* could bind collagen in a dose-dependent manner. The binding to collagen could be eliminated if the cells were pretreated with trypsin, demonstrating the presence of a proteinaeous receptor molecule. The binding to collagen could also be abolished if the cells were pre-incubated with rabbit antibody against AhsA, showing that AhsA is responsible for collagen binding.

The C-terminal 120 amino acids of AhsB were examined for their ability to form trimeric molecules, a property of trimeric adhesins. A fragment coding for the last 120 amino acids of AhsB, coding for a 19-kDa peptide, was cloned and expressed in *E. coli*. After SDS-PAGE electrophoresis, the 19-kDa monomer and a 57-kDa trimeric molecule were observed (unpublished). The ability to form stable trimers not denatured by SDS is a hallmark of these trimeric adhesins. Immunogold electron microscopy

showed limited labeling of gold particles on the cell surface. Control-cell samples showed no labeling; whereas cells pre-incubated with the rabbit anti-Ahs antiserum showed clusters of labeling. These data are not conclusive and may be due to low levels of the adhesin expressed under the culture conditions.

Together, these data suggest that *M. haemolytica* utilizes at least two adhesins, OmpA and Ahs, for attachment to fibronectin and collagen, respectively, which facilitate its attachment and colonization during an infection.

Genomic Analysis and Gene Regulation

Complete Sequence of M. haemolytica *A1*

A draft genome sequence of *M. haemolytica* strain BAA-410 (serotype 1), a bovine respiratory disease isolate, has been completed (Gioia et al. 2006). The genome consists of a circular DNA of 2.57 Mbp and contains 2839 coding sequences. Of these, 1966 have been assigned functions based either on previously published experimental data or on sequence homology. Comparison of the genomic sequence with those of other sequenced *Pasteurellaceae* species showed that *M. haemolytica* is most closely related to *Actinobacillus pleuropneumoniae* and *Haemophilus ducreyi*. Based on the sequences of 50 highly conserved housekeeping genes, *M. haemolytica, A. pleuropneumoniae*, and *H. ducreyi* form a group distinct from other *Pasteurellaceae* species. The high degree of similarity between *M. haemolytica* and *A. pleuropneumoniae* is not unexpected since both microorganisms are commensals of the upper respiratory tract and opportunistic pathogens of their respective hosts, bovine and porcine species. It is likely that they utilize similar pathogenic mechanisms in their respective hosts as reflected in their genomic similarities.

In Vivo *Gene Expression*

To date, virtually all studies with *M. haemolytica* have been carried out on cultures grown in the laboratory on rich media, with plenty of aeration and the optimal temperature of 37°C. This is the traditional method of preparing bacterial cultures but is clearly not the environment where the bacterium interacts with the host during an infection. Studies where an iron-chelating agent was added to the culture media to mimic iron-limiting conditions (Roehrig et al. 2007) have identified a number of iron-regulated proteins, some of which are likely expressed when the bacterium encounters iron limitation in the host. However, these conditions are also far from the natural environment.

Studies on *in vivo* gene expression in the host should provide a more accurate demonstration of the microbial activity in the host. With the available technology and sensitivity of polymerase chain reaction (PCR), it is possible to recover small quantities of bacterial mRNA from infecting cells for analysis directly or by microarray. Initial studies have been carried out on bacteria recovered directly from pneumonic lungs after an infection. Total RNA was isolated from these bacteria and used for reverse transcription (RT)-PCR analysis to study expression of a small panel of *M. haemolytica* genes. The data showed that there is a vast difference between *in vivo* gene expression compared with *in vitro* grown cultures (Lo et al. 2006). For example, when RNA from *in vitro*-grown cells was examined by RT-PCR, all genes examined showed a high level of expression. In contrast, when RNA from *in vivo*-grown cells was examined by the same RT-PCR, in general the level of gene expression was far lower than that *in vitro*.

Microarray Analysis

With the completion of the genome sequence, a microarray can be prepared to contain all of the predicted Orfs. Analysis of the array should present the overall gene expression profile. Roehrig et al. (2007) obtained approximately 90% of the genomic sequence of a *M. haemolytica* isolate and produced a microarray to examine gene expression profile when the bacterium was grown under iron-limited conditions. They identified the expression of genes coding for the transferrin receptor proteins and ABC-type transporters for uptake of hemoglobin, heme, and siderophores. They further characterized the expression of the hemoglobin receptors HmbR1 and HmbR2 by real-time PCR of *M. haemolytica*-infected lung tissue and showed that the relative levels of mRNA for *hmbR1 and hmbR2* were similar between the lung samples and the samples from the *in vitro* iron-limiting conditions.

Regulation of Gene Expression

Little is known about gene regulation in *M. haemolytica.* Even though many of the virulence genes have been characterized, there is little information on when these genes are expressed during an infection. As a commensal, one would expect that there are systems that allow the bacterium to sense its

environment and regulate expression of genes between the nasal location compared with the alveoli during an infection. One publication examined the expression of the *lkt* operon under iron-limited conditions and temperature (Strathdee and Lo 1989b). A report by Highlander suggested that a leucine finger is located upstream of the *lkt* promoter and may be involved in its regulation (Highlander and Hang 1997). Other studies have identified transferrin-binding protein gene expression in *M. haemolytica* under iron restriction (Deneer and Potter 1989).

One regulation system that has been investigated is the quorum-sensing system best illustrated in the marine bacterium *Vibrio harveyi*. The quorum-sensing system 2 in *V. harveyi* is an interspecies communication system and a similar system is present in over 30 different microorganisms (Bassler et al. 1997; Surette et al. 1999). One of the main components in system 2 is the LuxS protein involved in the synthesis of autoinducer-2 (AI-2). A *luxS* homolog has been identified and cloned from *M. haemolytica* (Malott and Lo 2002). This *luxS* homolog can complement a *luxS* mutant in *E. coli*, demonstrating its activity in the synthesis of AI-2. A *luxS* knock-out mutant has been constructed in *M. haemolytica* (van der Vinne et al. 2005). The mutant had a competitive disadvantage against the $luxS^+$ parent in calves. Additional components of the quorum-sensing system 2 pathway in the *M. haemolytica* genome have recently been identified. The *luxP, luxQ*, and *luxO* homologous genes are being cloned to examine their activity in the quorum-sensing pathway (unpublished data).

Recently, the role of two-component regulatory systems (TCS) in *M. haemolytica* has also been investigated. Two-component regulatory systems are involved in sensing environmental conditions and regulating gene expression in response (Stock et al. 2000). In many pathogens, TCS are involved in the regulation of expression of virulence genes (Bernardini et al. 1990; Cotter and Miller 1997). Based on the genomic sequence analysis, five complete TCS pairs have been identified in *M. haemolytica* (Inamoto and Lo 2008). This small number is likely due to the limited environments to which the bacterium is exposed. In contrast, there are over 30 systems in the *E. coli* genome, because of its much wider range of habitats. Most of the systems in *M. haemolytica* appear to be involved in sensing environmental changes, such as aerobic/anaerobic conditions.

The NarP/Q system has been selected for characterization (unpublished). A *narP* mutant of *M. haemolytica* exhibited an altered protein expression profile in response to $NaNO_3$ in comparison with the parental strain. A number of proteins with altered expression in the *narP* mutant have been identified. One of those proteins is FbpA, involved in iron uptake in response to iron deficiency. The FbpA promoter contains a number of NarP-binding sequences (Darwin et al. 1997), consistent with its regulation by NarP.

PATHOGENESIS AND DISEASE

Mannheimia haemolytica exits as part of the commensal nasopharynegeal microflora in healthy animals. Healthy animals are able to control multiplication of the bacterium in the nasopharynx. Further, small numbers of bacteria inhaled in aerosolized droplets are cleared by the host immune system. In stressed animals, *M. haemolytica* A1 can proliferate and multiply to high numbers in the nasopharynx and trachea, resulting in large numbers of bacteria being inhaled into and colonizing the lungs (Lillie and Thomson 1972). The process that results in the increase in *M. haemolytica* A1 in the nasal passage is not known but concurrent infection with other viral agents and stress, due to crowding, compromises the clearing mechanism in the lungs (Whiteley et al. 1992). The bacteria recovered from the lungs of animals that died from shipping fever were almost exclusively *M. haemolytica* A1 (Allan et al. 1985); although serotype A1 is not the dominant serotype in healthy animals. Therefore, there must be some unique property of serotype A1 that enables it to proliferate and colonize the lungs of stressed animals. A comparative analysis of the genomic sequences of several serotypes may identify differences that account for the success of serotype 1 during infection.

Bovine pneumonic pasteurellosis or shipping fever pneumonia is a major cause of sickness and death in the cattle industry in North America. As the name implies, the disease is often associated with shipping or transport of cattle over long distances. Typically, calves are born and raised on farms and ranches. At the age of 6–8 weeks, they are put into transport trucks or trains and shipped from the farm of origin to auction houses and sold to feedlots. The frequency and number of *M. haemolytica* isolated

from the nasal cavity of animals increase during the first 2 weeks after shipping (Thomson et al. 1969). When healthy animals from multiple sources are crowded together in an enclosed environment during transport, they are stressed; the condition provides an excellent environment for the transfer of infectious agents. These animals may carry and shed several respiratory viruses that can be transferred to other animals. Respiratory viruses such as bovine herpes virus and bovine viral diarrhea virus compromise the immune system, leading to subsequent infection and/or colonization by bacteria such as *M. haemolytica* inhaled from the nasopharynx (Frank et al. 1987). As a result of prior or concurrent viral infection, the animals cannot clear the bacteria that are inhaled into the lungs (Wilkie and Shewen 1983). These bacteria evade immune clearance, colonize the alveoli, and produce the molecules that cause damage to the host tissue, leading to pneumonia (Jensen et al. 1976).

Besides causing bovine pneumonic pasteurellosis, *M. haemolytica* also cause pneumonic disease in sheep and bighorn sheep. In fact, studies by Dassanayake et al. (2009) showed that the leukotoxin from serotype A1 appears to have target specificity toward leukocytes from bighorn sheep (see below).

Lesions

Macroscopic examination of the pneumonic lungs usually reveals fibrinous consolidation of between one-third to almost two-thirds of the anterior and cardiac lobes (Rehmtulla and Thomson 1981). In addition, pulmonary lesions consisting of fibrinous pleuritis associated with sheets of fibrin deposit or networks of fibrin on the visceral surfaces have been reported. Some researchers have reported the presence of fluid in the pleural cavity, as well as blood, pus, or mucus in the bronchi or bronchial tubes. Microscopic examination of pneumonic lesions in the lungs usually reveals extensive infiltration of neutrophils, polymorphonuclear cells, and macrophages into the pulmonary alveoli and alveoli ducts (Rehmtulla and Thomson 1981). In addition, there is also distension of the lymphatic vessels by both neutrophils and polymerized fibrin (Ackermann and Brogden 2000). Some areas of coagulation necrosis may develop into liquefaction necrosis (Friend et al. 1977). The bronchi and bronchioles in the lobes exhibit varying degrees of inflammation and are often filled with a purulent exudate containing mostly degenerating mononuclear cells and necrotic debris. Morbidity is high, with significant weight loss and ultimately possible death of the animal.

Immunity and Vaccination

Vaccines based on whole cell bacterins of *P. haemolytica* have been in use since the mid-1950s. However, they were not protective and instead immunization sometimes correlated with more severe pneumonia (Panciera et al. 1984). Subsequently, antigen-based vaccines that confer adequate protection have been developed. These vaccines were primarily based on cell-free crude culture concentrates (Durham et al. 1986; Shewen and Wilkie 1988). One of these successful culture supernatant vaccines is called Presponse (Shewen et al. 1988; Jim et al. 1988).

An attempt to improve the culture supernatant vaccine was made by supplementation of the vaccine with recombinant leukotoxin (rLkt; Conlon et al. 1991). The results showed that this could improve efficacy by approximately 30%. Unfortunately, no further development with this approach has been made.

A novel approach to produce an edible vaccine in transgenic plants for oral delivery has been investigated. An edible vaccine for oral delivery would be an advantage for the vaccination of a large herd of animals as they could be fed the vaccine (plant material) instead of requiring individual needle injections. A vaccine produced in transgenic plants would have no requirement for animal products, processing or extraction, or refrigeration.

For *M. haemolytica*, a number of genes encoding antigens have been cloned and transformed into clover and alfalfa by *A. tumefaciens*-mediated transformation. A derivative of the leukotoxin Lkt50 was expressed as a fusion protein with green-fluorescence protein (GFP) both in clover and alfalfa (Lee et al. 2001). Lkt50 lacks the hydrophobic regions of the toxin and therefore cannot insert into target cells. The Lkt50-Gfp fusion protein was stably expressed in clover and alfalfa. When an extract from clover-containing Lkt50-Gfp was used to immunize rabbits via needle injection, the rabbits produced antibodies that neutralized the authentic leukotoxin from *M. haemolytica*. A method was also developed to dry the harvested plant materials by heating the plants at 50°C for 2 days. The dried material still contains the Lkt50-Gfp protein after prolonged storage for over 2 years at room temperature (Lee et al. 2003).

The gene coding for another antigen GS60 was also transformed into alfalfa as a Gfp fusion (Lee et al. 2008). Similar to Lkt50-Gfp, the GS54-Gfp protein from alfalfa was immunogenic by needle injection into rabbits and induced antibodies that recognized the authentic Gs60 antigen from *M. haemolytica*.

In order to measure any level of immune response in cattle, the initial vaccination (feeding) trials were carried out in colostrum-deprived calves. After several feeding trials, the data showed that calves fed Lkt-alfalfa tended to exhibit a lower clinical score of pneumonia than calves fed Gfp-alfalfa (unpublished data). In addition, the lungs from Lkt-alfalfa-fed calves showed no evidence of pneumonia. This suggests that the calves exhibited a slight degree of protection from the oral vaccine. However, due to the low number of animals used in each trial, as well as animal to animal variations, no statistical analysis could be performed on the data.

A larger scale feeding trial was carried out on conventional calves. In this study, a herd of 20 calves was fed Lkt-alfalfa and 10 calves were fed Gfp-alfalfa following similar procedure with the colostrum-deprived calves. The main difference was in the challenge where a higher dose of 10^9 CFU/ml of *M. haemolytica* was used. After challenge, the calves were again monitored for pneumonic scores. Nasal secretion samples were collected and assayed for IgA antibodies against the authentic leukotoxin. Preliminary results showed that animals fed Lkt-alfalfa exhibit a higher level of anti-Lkt IgA antibodies (unpublished data).

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Research on bovine pneumonic pasteurellosis has improved our understanding of the involvement of *M. haemolytica* A1 as the central causative agent of the disease. Many of the virulence factors and antigens of *M. haemolytica* A1 that contribute either directly or indirectly to the disease have been characterized. The genes coding for these molecules have also been cloned and sequenced. The completion of the *M. haemolytica* A1 genomic sequence provides a framework for future studies on additional virulence factors and antigens. On the other hand, the mechanisms that regulate expression of these virulence factors have not been determined. Studies on gene regulation systems as well as response to environmental changes/signals should be a focus of future research.

Since most studies have been carried out on laboratory-grown cultures, there is uncertainty about the extent to which observations from the *in vitro* studies reflect *in vivo* conditions. Therefore, it is important to examine expression of the virulence genes during infection in the host to obtain a true picture of the role(s) of the virulence factors during infection. It is anticipated that some virulence genes are expressed during the early stages of infection before the animal exhibits any clinical signs of pneumonia. These virulence factors could be involved in the early stages of colonization and evasion of the host immune response. Virulence factors expressed in later stages of the infection could be involved in sustaining survival and destruction of host tissue.

With respect to vaccination and protection against *M. haemolytica* A1, the current antigen-based vaccines have been quite effective. Improvement in efficacy as well as delivery of the vaccine may be achieved with the development of an edible vaccine that includes several of the key protective antigens, reducing the cost of vaccine production and possibly increasing efficacy and delivery. In addition, specific antigens from other *M. haemolytica* serotypes and respiratory pathogens, bacterial or viral, could be incorporated into the oral delivery system to make it a more comprehensive vaccine.

Finally, little work has been conducted on the response of animals to lung invasion by *M. haemolytica* A1. Little is known about the host immune response to the presence of the bacterium inhaled into the alveoli. The immune response, in the form of cytokines and inflammatory molecules, may trigger additional gene expression from the bacterium. The completion of the bovine genome should facilitate the development of arrays for expression and proteomic studies from the host. These data could be used to further examine gene expression from *M. haemolytica* A1 when faced with these host molecules.

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19
Actinobacillus

J. I. MacInnes

Members of the genus *Actinobacillus* are small, gram-negative, pleomorphic, coccobacillary rods that are facultatively anaerobic, indole negative, β-galactosidase, and urease positive, and they reduce nitrates. Apart from *Actinobacillus pleuropneumoniae* and some *Actinobacillus suis* isolates, most *Actinobacillus* isolates grow on MacConkey's agar. The optimum temperature for growth is 37°C, and all have complex nutritional requirements (Mannheim et al. 1984; Phillips 1984). These bacteria are associated with mucous membranes, and the host range of different species tends to be limited. Many are associated with farm animals, but *Actinobacillus hominis* and *Actinobacillus ureae* are opportunistic pathogens of man.

Historically there was considerable confusion about the classification of *Actinobacillus* species and other members of the family *Pasteurellaceae* because assignments were made on the basis of phenotypic characteristics such as the need for nutritional supplements or serological cross-reactivity. Although recent taxonomic studies have employed a variety of molecular methods, proper speciation of these organisms remains a challenge. Christensen and Bisgaard (2004) suggested that of the 22 species (or species-like taxa) that had been described as belonging to the genus *Actinobacillus, Actinobacillus sensu stricto* should be restricted to *Actinobacillus lignieresii*, *A. pleuropneumoniae*, *Actinobacillus equuli* subsp. *equuli*, *A. equuli* subsp. *haemolyticus* (taxon 11 of Bisgaard), *A. hominis*, *A. suis*, *A. ureae*, *Actinobacillus arthritidis* (taxon 9 of Bisgaard), *Actinobacillus* genomospecies 1 and 2, and taxa 8 and 26 of Bisgaard. They suggested that the remaining 11 species (a*ctinomycetemcomitans*, *capsulatus, dephinicola, indolicus, minor, muris, porcinus, rossii, scotiae, seminis*, and *succinogenes*) "are unrelated to *A. sensu stricto* and should consequently be grouped with other genera or be renamed as new genera depending on new data." Since that landmark paper, *Actinobacillus actinomycetemcomitans* has been reclassified as *Aggregatibacter actinomycetemcomitans* and the descriptions of *Actinobacillus capsulatus* and [*A.*] *rossii* have been emended (Christensen et al. 2005; Nørskov-Lauritsen and Kilian 2006; Kuhnert et al. 2007). In addition, "*Actinobacillus porcitonsillarum*" a nonpathogenic tonsillar isolate that is phenotypically very similar to *A. pleuropneumoniae* has been described, which can only be reliably identified by polymerase chain reaction (PCR) testing (Tonpitak et al. 2007). Despite the uncertainty with classification of *Actinobacillus* species, Dousse et al. (2009) have recently described a routine phenotypic identification scheme that can be used to easily identify members of the genus that are of interest in the veterinary diagnostic laboratory, including *A. lignieresii*, *A. pleuropneumoniae* (biovars I and II), *A. suis*, *A. equuli* (subsp. *equuli* and *haemolyticus*), [*A.*] *rossii*, [*A.*] *minor*, [*A.*] *porcinus*, *A. capsulatus*, and "*A. porcitonsillarum.*"

A summary of important *Actinobacillus* and *Actinobacillus*-like species found in animals is presented in table 19.1.

Table 19.1. Habitat, Primary Host, and Diseases of Selected *Actinobacillus* Spp. Found in Animals

Species	Main habitat	Primary host[a]	Pathogenic potential, disease
A. arthritidis	Oral cavity?	Horses	Opportunistic pathogen, septicemia
A. capsulatus	Upper respiratory tract	*Leporidae*	Arthritis, septicemia
A. equuli subsp. *equuli* and *A. equuli* subsp. *haemolyticus*	Upper respiratory and intestinal tract	Horses	Commensal and opportunistic pathogen in foals (sleepy foal disease, joint ill purulent nephritis, and arthritis) and adults (abortion, septicemia, nephritis, endocarditis)
A. lignieresii	Upper respiratory and intestinal tracts	Cattle	Commensal and opportunistic pathogen (wooden tongue)
A. pleuropneumoniae	Upper respiratory tract	Pigs	Primary pathogen,[b] pleuropneumonia
A. suis	Upper respiratory tract	Pigs	Commensal and opportunistic pathogen in piglets (primarily septicemia) and adults (septicemia, pneumonia, arthritis, enteritis)
[A.] *indolicus* [A.] *minor* [A.] *porcinus*	Upper respiratory tract	Pigs	Commensal
[A.] *rossii*	Urogenital tract	Pigs	Opportunistic pathogen, reproductive tract infections
[A.] *seminis*	Urogenital tract	Sheep	Opportunistic pathogen, epididymitis
[A.] *succinogenes*	Intestinal tract	Cattle	Commensal, produces succinic acid used indirectly by the host?
"*A. porcitonsillarum*"	Tonsils	Pigs	Commensal, easily confused with *A. pleuropneumoniae,* [A.] *minor*

[a] Many opportunistic pathogens of the genus can cause sporadic disease in other species.
[b] Some serotypes in some animals may behave as commensals.

ACTINOBACILLUS PLEUROPNEUMONIAE

Epidemiology

Two biotypes of *A. pleuropneumoniae* that are differentiated based on their requirement for nicotinamide adenine dinucleotide (NAD; table 19.2) have been described . Biotype 1 strains require NAD; whereas biotype 2 strains can synthesize NAD in the presence of specific pyridine nucleotides or their precursors (Niven and Lávesque 1988). Although there are reported exceptions, 13 serovars (1 to 12, and 15) are biotype 1; whereas most serovar 13 and 14 isolates are biotype 2.

To date, 15 serovars of *A. pleuropneumoniae* have been described based on their capsular polysaccharide antigens (Blackall et al. 2002). Serovars 1 and 5 have been further differentiated into la and 1b and 5a and 5b, respectively, based on minor differences in the polysaccharide structure, but such subtyping is not usually done in diagnostic laboratories. The chemical structure of all but the K14 capsule has been reported (Perry et al. 2005). Usually, a given K type is associated with a particular O-antigen type, but exceptions have been reported (Gottschalk and Taylor 2006). Cross-reactivity among some serotypes has been reported. Identical O-antigens are found in K1 and 9 and in

K3, 8, and 15 strains, and there are antigenically similar O-antigens in K1, 9, and 11; K3, 6, 8, and 15; and K4 and 7 strains (Dubreuil et al. 1990; Perry et al. 2005; table 19.2).

Three capsule groups have been described based on structural differences. The capsular polysaccharide (CPS) of Group I *A. pleuropneumoniae* capsules found in serovars 1, 4, 12, and 15 are composed only of repeating oligosaccharide linkages joined by phosphate linkages; whereas Group III capsules, present in serovars 2, 3, 6, 7, 8, 9, 11, and 13, are composed of teichoic acid polymers joined through phosphate diester linkages. Group II CPS found in serovars 5 and 10 is comprised of repeating oligosaccharides only. Knowledge of the detailed structure of *A. pleuropneumoniae* cell surface polysaccharides has been a key for the development of serological tests to detect infection, for serotyping methods used in strain characterization, and for epidemiological studies.

Although PCR-based tests for "serotyping" based on both capsule and *apx* toxins have been described, conventional serological methods such as slide agglutination, complement fixation, and indirect hemagglutination are still used most often and many would consider them the "gold standard" (Rayamajhi et al. 2005; Zhou et al. 2008). In addition, a number of different techniques have been reported for use in more detailed strain identification including restriction endonuclease fingerprinting, ribotyping, pulsed-field gel electrophoresis (PFGE), and PCR restriction fragment length polymorphism (RFLP) (Kokotovic and Angen 2007). With most of these methods, clinical isolates of the same serovar appear to be quite homogeneous.

Pleuropneumomia often occurs as epizootic but is rapidly becoming endemic in many countries. Detection of subclinically infected animals is difficult by culture even with selective media. Immunomagnetic methods improve detection, but PCR-based tests, especially post-culture, are most sensitive. Increasingly, enzyme-linked immunosorbent assays for long-chain O-antigens or the ApxIV toxin are used for routine epidemiological surveys. This information is important for controlling the spread of the disease through herd management practices and vaccination.

Actinobacillus pleuropneumoniae is common in all countries where pigs are intensively reared. A limited number of serovars tend to be reported in a given country or region, but low-virulence serovars may be overlooked. For example, in a serological survey of Canadian swine, antibodies could be detected to serovars 1-9-11; 3-6-8-15, 4-7, and 12 (MacInnes et al. 2008). The picture is also made complicated by the fact that some serovars or strains may be more virulent in some countries than in others (Gottschalk and Taylor 2006). Unfortunately, there have been relatively few recent publications regarding the prevalence of different *A. pleuropneumoniae* serovars. Traditionally, the most prevalent serovars in Asia and Australia were 1, 2, and 5. In Great Britain and Europe, serovars 2 and 9 were found most often, whereas in Canada, the United States, and Mexico, serovars 1 and 5 predominated. Although there are usually 1 to 3 predominant serovars, as many as 11 different serovars have been reported in some countries (Dubreuil et al. 2000). More recently, serovar 15 has been reported in Australia and Japan, while serovars 1, 3, 4, 5, and 7 predominate in China (Blackall et al. 2002; Xu et al. 2008).

Eleven serovars are capable of causing pleuropneumonia, although some serovars are thought to be more virulent than others. These differences may be attributed, at least in part, to the production of different combinations of the Apx toxins (table 19.3), with the most virulent serovars producing both ApxI and ApxII. Other factors, notably the amount of surface polysaccharide present, may also contribute to differences in virulence both between serovars and between strains within the same serovar.

Transmission and Disease

Actinobacillus pleuropneumoniae is considered an obligate parasite of the porcine respiratory tract. The organism can be isolated from nasal cavities, tonsils, and lungs of infected pigs. It is not normally considered an invasive bacterium, although there is one report of *A. pleuropneumoniae* being recovered from cases of osteomyelitis in pigs. There are no other known natural hosts, and the organism does not survive for long in the environment (Gottschalk and Taylor 2006). The organism is present in nasal secretions and lung exudates of acutely infected animals and transmission is by aerosols or direct contact with infected pigs.

Pleuropneumonia can occur in pigs of all ages. Increased incidence of pleuropneumonia is associated with conditions such as overcrowding, poor ventilation, and large temperature fluctuations (Gottschalk and Taylor 2006). Many studies suggest

Table 19.2. Properties and Distribution of *A. pleuropneumoniae* Serovars

Serovar	K antigen	Capsule group	O antigen[a]	Biotype	Toxins[b]	Virulence potential[c]	Country, region[d]
1	K1	I	O1[a]	1	ApxI, ApxII, ApxIV	High	Widespread
2	K2	III	O2	1	ApxIIA, ApxIII, ApxIV	Low → high	Europe
3	K3	III	O3[b]	1	(ApxII)*e* ApxIII, ApxIV	Low → high	China, Europe
4	K4	1	O4[c]	1	ApxII, ApxIII, ApxIV	Low	Spain
5	K5	II	O5	1	ApxI, ApxII, ApxIV	High	Widespread
6	K6	III	O6	1	ApxII, ApxIII, ApxIV	Low	Widespread
7	K7	III	O7	1	ApxII, ApxIV	High	Canada, Europe
8	K8	III	O3	1	ApxII, ApxIII, ApxIV	Low	Mexico, United Kingdom
9	K9	III	O9	1	ApxI, ApxII, ApxIV	High	Europe
10	K10	II	O10	1	ApxI, ApxIV	Very high?	Europe
11	K11	III	O1	1	ApxI, ApxII, ApxIV	High	Netherlands
12	K12	1	O12	1	ApxII, ApxIV	Low	Europe, Canada
13	K13	3	O7	2	ApxII, ApxIV	Low	Europe
14	"K14"	ND[d]	O14	2	ApxI, ApxIV	Low	Europe
15	K15	1	O3	1	ApxII, ApxIII, ApxIV	High	Australia, Japan

[a] Several O-antigens are structurally and antigenically very similar, for example, O9 and O1; O6 and O3; O7 and O4.
[b] The *apxIBD* genes are found in all serotypes except serotype 3. As a result, ApxII is not exported.
[c] The virulence can vary markedly from country to country; for example, serovar 3 strains are associated with disease in Asia, but are considered benign in Europe; European serovar 2 strains are more virulent than serovar 2 isolates that are occasionally isolated in North America.
[d] The structure of the K14 antigen has not been reported.

Table 19.3. Apx Toxins of *Actinobacillus pleuropneumoniae*

	Genes			Activities		Size (kDa)	Serovar
Toxin	Activator	Structural	Export	Hemolytic	Cytotoxic		
ApxI	*apxIC*	*apxIA*	*apxIBD*	strong	weak	110[a]	1, 5, 9, 10, 11, and 14
ApxII	*apxIIC*	*apxIIA*	*apxIIBD*	weak	moderate	103	All but 10 and 14
ApxIII	*apxIIIC*	*apxIIIA*	*apxIIIBD*	none	strong	112	2, 3, 4, 6, 8, and 15
ApxIV	*apxIVC*	*apxIVA*	?	weak	?	202[b]	All

[a]Although the predicted size of the ApxI toxin is 110 kDa, on SDS polyacrylamide gels, it runs much closer to the ApxIIA than to ApxIIIA.
[b]Deduced size of ApxIV in the Shope 4074 strain; the number of glycine-rich repeats (and hence size) varies in other serovars.

that there is no association with predisposing viral or bacterial infections; however, *Mycoplasma hyopneumoniae* has been shown to potentiate *A. pleuropneumoniae* disease in experimentally infected specific pathogen-free pigs (Fraile et al. 2009; Marois et al. 2009).

The pace of disease can range from peracute to chronic depending on the serovar, the immune status of the host, and the number of bacteria reaching the lung. During peracute or acute disease, pigs may exhibit some or all of the following clinical signs: high fever, increased respiratory rate, coughing, sneezing, dyspnea, anorexia, ataxia, vomiting, diarrhea, and severe respiratory distress with cyanosis (Gottschalk and Taylor 2006).

Pulmonary lesions associated with peracute and acute disease are characterized by severe edema, inflammation, hemorrhage, and necrosis (Bertram 1988). The thoracic cavity is often filled with serosanguinous fluid and fibrin clots, and diffuse fibrinous pleuritis and pericarditis are common. In the early stages of disease, marked polymorphonuclear leukocyte (PMN) infiltration, edema, and fibrinous exudate are apparent. In the later stages, macrophage infiltration is more apparent, and necrotic areas are surrounded by dense bands of degenerating leukocytes. Severe necrotizing vasculitis leads to hemorrhage in the lung. *A. pleuropneumoniae* can be found within alveolar and interlobular fluid and may spread from the parenchyma to the pleura via lymph vessels of interlobular septa and pleura, but bacteremia is rare. *In situ* hybridization has revealed large numbers of phagocytosed *A. pleuropneumoniae* within alveolar macrophages and PMNs and has confirmed that this bacterium does not invade epithelial cells. Animals that survive infection may have complete resolution of lesions, but frequently they retain focal necrotic sequestra and/or well-encapsulated abscesses with overlying areas of fibrinous connective tissue. Chronically infected animals may also harbor *A. pleuropneumoniae* in tonsillar crypts (Bossé et al. 2002).

Pathogenesis of *Actinobacillus pleuropneumoniae* Infection

There are three basic stages in the pathogenesis of acute pleuropneumonia: colonization, evasion of host clearance mechanisms, and damage to host tissues. While a complete picture of the pathogenesis of *A. pleuropneumoniae* infection has not yet emerged, some of the factors known to contribute to each of these stages are discussed below.

Colonization

Adhesins. Colonization is often a prerequisite to the production of disease. Although *A. pleuropneumoniae* can be isolated from the tonsils and nasal cavities of pigs, it binds preferentially to cells of the lower respiratory tract (Dom et al. 1994). However, it remains to be established whether colonization of the upper respiratory tract is a necessary prelude to pulmonary infection in naturally occurring cases of pleuropneumonia. In infection studies with gnotobiotic piglets, *A. pleuropneumoniae* cells are initially mainly associated with the stratified squamous epithelium and detached epithelial cells while at later times, *A. pleuropneumoniae* is found closely associated with the crypt walls and with detached cells present in the crypts (Chiers et al. 1999). There are also data to suggest that *A. pleuropneumoniae* can

bind to low-molecular-weight proteins in respiratory tract mucus (Bélanger et al. 1994).

Several lines of investigation have pointed to an important role for lipopolysaccharide (LPS) in adherence to the host. As noted above, several different O types of *A. pleuropneumoniae* have been described (table 19.2). All serovars of *A. pleuropneumoniae* studied to date appear to have the same inner core structure consisting of a trisaccharide of l-*glycero*-d-*manno*-heptose residues linked to a Kdo residue, where the proximal heptose residue (Hep I) is substituted at the 3-position by the second l-*glycero*-d-*manno*-heptose residue (Hep II) of the l-*glycero*-d-*manno*-heptose trisaccharide, at the 4-position by a β-glucose residue (Glc I), and at the 6-position by an α-glucose residue (Glc II; Michael et al. 2004). There is a d-*glycero*-d-*manno*-heptose residue (Hep IV) at the 6-position of the Glc I residue, and a l-*glycero*-d-*manno*-heptose residue (Hep III) at the 2-position of Hep II completing the conserved inner core oligosaccharide (OS) structure. Genes involved in core synthesis can be detected in serovars 1 to 12, again suggesting that there is a conserved inner core (Jacques 2004). There are at least two types of outer core structure in *A. pleuropneumoniae*. In serovar 1, Hep IV of the inner core is alternatively substituted at the 4-position by a trisaccharide of (1*S*)-GalNAc-(1 → 4,6)-α-Gal-(1 → 3)-β-Gal-, where the GalNAc residue has an unusual open-chain configuration while a second d-*glycero*-d-*manno*-heptose residue (Hep V) substitutes Hep IV at the 6-position in serovars 2, 5a, and 5b. In the case of serovar 2, there is an additional glucose residue linked to the 4-position of the Hep IV residue (Michael et al. 2004). Data by Jacques (2004) and others suggest that the O-oligosaccharides of *A. pleuropneumoniae* might be used to form a low affinity interaction with host molecules such as phosphatidylethanolamine while core oligosaccharides together with other cell surface proteins (see below) might interact more avidly. Work with capsule-deficient mutants suggest, however, that core and other adhesins can be blocked in the presence of capsule (Rioux et al. 2000).

van Overbeke et al. (2002) demonstrated that expression of type 4 fimbriae and a 55-kDa outer membrane protein (OMP) is associated with high levels of adherence. In this study, growth under NAD-restricted conditions was necessary to observe optimal adherence and expression of both fimbriae and the 55-kDa OMP in serovars 5a, 9, and 10 while growth conditions had no effect on the serovar 2 strain tested. In later work by Boekema et al. (2004), fimbrial genes were not expressed in standard laboratory media, but could be detected when the cells were grown in chemically defined medium or were put in contact with primary lung epithelial cell cultures. Although the role of the 55-kDa protein is uncertain, fimbriae are likely involved in adherence of *A. pleuropneumoniae* at some point during the disease process.

There are also data to suggest that *A. pleuropneumoniae* can bind to extracellular matrix components. Studies by Enríquez-Verdugo et al. (2004) have shown that a 60-kDa OMP present in most but not all serovars can interact with swine collagen types I, III, IV, and V in a Ca^{2+}- dependent fashion. Given the abundance of collagen in the swine lung parenchyma, it is possible that the 60-kDa OMP is important in the pathogenesis of *A. pleuropneumoniae*, but further experiments are needed to demonstrate this conclusively. *A. pleuropneumoniae* can also interact with fibronectin. Like other members of the family *Pasteurellaceae*, *A. pleuropneumoniae* has a ComE1 fibronectin-binding protein (Mullen et al. 2008). In addition to binding fibronectin, this small protein can bind double-stranded DNA in a sequence-independent manner, and inactivation of the *comE1* gene in an *A. pleuropneumoniae* serovar 15 strain resulted in a 10^{-4}-fold drop in transformation efficiency.

In studies by Auger et al. (2009), 2 of 14 genes that are part of a *tad* locus were found to be up-regulated upon contact with porcine lung cells. In *A. actinomycetemcomitans,* genes in the *tad* locus are associated with nonspecific adherence to both biotic and abiotic surfaces and it is likely that the products of the *tad* locus in *A. pleuropneumoniae* (which is very similar to that in *A. actinomycetemcomitan*) play a similar role. Moreover, it is possible that expression of the *tad* genes leads to the rough phenotype frequently seen with *A. pleuropneumoniae* following primary isolation. In addition to these genes, a homolog of the gene for the *Haemophilus influenzae* autotransporter adhesin Hsf and two genes involved in poly-β-1,6-N-acetyl-D-glucosamine (PGA) biofilm formation (*pgaB* and *pga*) have also been found to be up-regulated following contact with porcine lung cells. The ability of *A. pleuropneumoniae* to form biofilms is very common; this phenotype can be rapidly and permanently lost after subculture so for many years was

overlooked (Kaplan and Mulks 2005). Although the picture is far from complete, it appears that many factors regulate the genes involved in biofilm formation (Dalai et al. 2009), but based on the findings of Buettner et al. (2009), biofilm production is likely up-regulated under anaerobic conditions.

An autotransporter protease, AasP, has also been described in *A. pleuropneumoniae*, but its role in pathogenesis is uncertain. In aerosol challenge studies in pigs, a serovar 15 *aasP* mutant lacking 123 bp spanning the predicted catalytic site adhered more tightly than wild type under standard and NAD-restricted growth conditions, but less tightly under iron-restricted conditions. No significant differences between AP76 wild type and the AP76 Δ*aasP* mutant were observed, suggesting that AasP does not play a crucial role in *A. pleuropneumoniae* virulence (Tegetmeyer et al. 2009). It is tempting to speculate that AasP might be involved in orchestrating the processing of some adhesins, but to date, OmlA is the only protein that AasP has been shown to process, and this protein has no demonstrated role in attachment or biofilm formation.

In silico work by Chung et al. (2007) predicts that 45 OMPs and 48 lipoproteins might be present on the *A. pleuropneumoniae* cell surface so it would not be surprising to find even more adhesins that might be involved in colonization and pathogenesis.

Very little work has been done on the identification of host cell receptors, but Abul-Milh et al. (1999) showed that *A. pleuropneumoniae* whole cells, LPS, and detoxified LPS can bind to glucosylceramide (Glcβ1Cer), galactosylceramide (Galβ1Cer) with hydroxy and nonhydroxy fatty acids, sulfatide (SO(3)-3Galβ1Cer), lactosylceramide (Galβ1-4Glcβ1Cer), gangliotriaosylceramide GgO3 (GalNAcβ1-4Galβ1-4Glcβ1Cer), and gangliotetraosylceramide GgO4 (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1Cer) glycosphingolipids. In these studies, binding to GlcCer, GalCer, sulfatide, and LacCer, but not to GgO3 and GgO4 glycosphingolipids, was inhibited by monoclonal antibodies against LPS O-antigen, suggesting involvement of LPS in recognition of GlcCer and LacCer, where glucose is probably important for LPS binding; GalCer and sulfatide glycosphingolipids, where the sulfate group is part of the binding epitope; and GgO3 and GgO4, where GalNacβ1-4Gal disaccharide is the minimal common binding epitope.

Nutrient Acquisition. Establishment of infection depends on the ability of the bacterium to acquire all essential nutrients for growth. Within the respiratory tract environment, the variety and quantity of available carbohydrates and certain other nutrients are restricted (Macfadyen and Redfield 1996). The ability to overcome nutritional limitation within the host may be considered a virulence factor.

Almost all bacteria require iron for survival at concentrations of $\sim 10^{-7}$ M. Iron is needed for the function of enzymes involved in a large number of essential functions (e.g., ATP generation, respiration, DNA replication and repair) and for regulatory molecules such as HlyX. In the host, free iron concentrations may be as low as 10^{-18} M as almost all of it is tightly bound by molecules such as transferrin, lactoferrin, haptoglobin, and hemoglobin. *A. pleuropneumoniae* has a surprising number of different iron-acquisition systems. It can use heme compounds (including free heme, hemin, hematin, and hemoglobin) as well as porcine transferrin, but not lactoferrin, for growth. In addition, *A. pleuropneumoniae* can utilize ferrichrome and the bis-catechol siderophore ISD-I 207 (Shakarji et al. 2006).

A ferric uptake regulator (Fur) protein has been described in *A. pleuropneumoniae* (Jacobsen et al. 2005a). As in other bacteria, iron-loaded Fur interacts with Fur box sequences in promoters of a number of iron-regulated genes repressing transcription. Fur might also be involved in the positive regulation of some genes, but that remains to be demonstrated in *A. pleuropneumoniae*. Deletion of the *fur* gene has a pleotrophic effect. *Fur* mutant strains constitutively express transferrin-binding proteins and grow less well than wild type on a variety of different media *in vitro*, perhaps due to accumulation of toxic levels of iron. Also, *fur* mutants are attenuated *in vivo*. Again, this could be due to a slower growth rate than wild type or more rapid elimination of the mutant by the host. This greater host response may be due, at least in part, to the constitutive expression of the highly immunogenic transferrin-binding proteins. Also, it is possible that there are lower levels of expression of ApxII or other virulence factors, but further work is needed to confirm this.

Actinobacillus pleuropneumoniae makes an iron-repressible transferrin-binding system that binds porcine transferrin, but not bovine, ovine, avian, or human transferrin (Jacques 2004). The auxillary molecule, TbpB (TfbA), is a lipoprotein of about

60-kDa while the 100-kDa TbpA (TfbB) protein is thought to form a transmembrane channel. Iron is likely removed from transferrin at the bacterial surface by the coordinate action of TbpA and TbpB followed by transport across the outer membrane via TbpA and binding of iron by a periplasmic-binding protein. *A. pleuropneumoniae* serovar 7 mutants with deletions in *tbpB*, *tbpA* or both genes are are cleared rapidly and are avirulent in an aerosol infection model (Baltes et al. 2002). The transferrin-binding proteins are highly immunogenic and antibodies to the 60-kDa protein can provide a protective but serotype-specific response.

All serovars of *A. pleuropneumoniae* are capable of obtaining heme products via production of hemolysins (Frey et al. 1993). A Fur-regulated 105-kDa hemoglobin-binding receptor, HbgA has also been described in *A. pleuropneumoniae;* homologs of the *hgbA* gene are found in all serovars (Shakarji et al. 2006). In addition to binding hemoglobin, HbgA also likely binds hemin. Consistent with this notion, *in silico* modeling work by Pawelek and Coulton (2004) suggests that loops 2 and 7 of HgbA could be responsible for the recognition and binding of hemoglobin or heme ligands. Although *hgbA* mutants are able to infect specific pathogen-free piglets by the aerosol route, clinical signs and lung lesion scores are much less than those expected of the parental strain (Shakarji et al. 2006).

Actinobacillus pleuropneumoniae also makes a ferrichrome-specific receptor encoded by the *fhuC*, *DB*A operon, which, unlike the other iron uptake systems, is not iron-regulated (Mikael et al. 2002). The FhuA protein is a 77-kDa OM receptor for ferrichrome, while FhuD (35.6 kDa) is a periplasmic protein responsible for the translocation of ferric hydroxamate from the outer to the inner membrane; FhuC (28.5 kDa) and FhuB (69.4 kDa) form a cytoplasmic membrane-associated ABC transporter that internalizes the ferric hydroxamate (Jacques 2004). Although the FhuA system is found in all serovars of *A. pleuropneumoniae*, challenge experiments indicate that *fhuA* mutants are just as virulent as wild type, suggesting that this iron uptake system does not play a key role in virulence. Moreover, it would appear that there is another siderophore-binding system that can use one or more catechol siderophores. In microarray studies by Deslandes et al. (2007), a putative enterochelin-like siderophore receptor, a potential Yfe system for the acquisition of chelated iron, a putative hemoglobin acquisition system homologous to the *Neisseria meningitidis* HmbR system, and a putative Fe^{2+}-specific porin system were found to be up-regulated during iron limitation. The precise roles of these genes in virulence and iron uptake remain to be established, however.

Release of the strongly bound iron complexes from high-affinity OM receptors and subsequent transport across the OM requires energy (Braun and Killmann 1999). As with many other bacteria, a TonB system, which transduces the proton motive force from the cytoplasmic membrane, is used to energize iron uptake at the outer membrane. In *A. pleuropneumoniae*, the *tonB1-exbB1-exbD1* system is transcriptionally linked to the *tbpA-tbpB* genes. A second TonB cluster (*exbB2-exbD2-tonB2*), which is highly homologous to others found in members of the family *Pasteurellaceae*, has also been described (Beddek et al. 2004). The expression of both *tonB2* and *tonB1* is up-regulated by iron restriction. TonB2, however, appears to play a much more important role in *A. pleuropneumoniae* virulence than TonB1. In an acute porcine infection model, Beddek et al. (2004) found that a *tonB2* mutant was highly attenuated, while a *tonB1* mutant was not. Moreover, TonB2, but not TonB1, is needed for growth when the sole source of iron is hemin, porcine hemoglobin, or ferrichrome. Neither *tonB* mutant is viable when transferrin is the sole iron source. In the case of the *tonB1* mutant, this is likely the result of the polar effect of the mutation on transcription of downstream *tbp* genes; in the case of the *tonB2* mutant, it appears that accessory proteins of the TonB1 system are needed for its function. Based on these findings, Beddek et al. (2004) suggest that the *tonB1-tbp* gene cluster confers a biological advantage by allowing the uptake of transferrin iron, but TonB1 itself plays little or no part in this process. The importance of the *exbB* gene linked to *tonB1* has also been demonstrated by the fact that a serovar 7 *A. pleuropneumoniae* Δ*exbB* mutant is unable to cause disease or colonize sufficiently long to initiate a detectable humoral immune response (Baltes et al. 2001).

Two ABC-transport systems involved in the uptake of iron across the cell membrane have been described in *A. pleuropneumoniae*. The FhuBCD system is specific for ferric hydroxamate while the AfuABC system is likely responsible for the transport of iron obtained from other sources. The details for the release of iron are not known, but permeases specific for both unchelated Fe^{3+} as well

as various iron complexes have been described in other systems (Braun and Killmann 1999).

Iron is not the only limited nutrient within the respiratory tract environment that may affect the ability of *A. pleuropneumoniae* to survive and cause disease. An ABC-transport operon *(cbiKLMQO)* that appears to be required for high-affinity nickel uptake has also been described (Bossé et al. 2001). Nickel is required for urease activity in *A. pleuropneumoniae* and, like iron, is only available in extremely low concentrations within mammalian hosts. A mutant with a transposon insertion within the *cbiK* gene was urease-negative under nickel-limiting conditions and unable to establish infection following low-dose aerosol challenge (Bossé and MacInnes 2000, 2001). The contribution of nickel to the virulence of *A. pleuropneumoniae* is likely via its role in urease activity since a mutation in the urease operon led to a similar level of attenuation (Bossé and MacInnes 2000). Nevertheless, it is possible that nickel is required for other functions in this bacterium.

As revealed in signature-tagged mutagenesis studies, many nutrient uptake systems such as ABC transporters for uptake of polyamines, certain sugars, and trace metals appear to be required for survival within pig lungs. In addition, urease activity could play a role in nutrient acquisition via the production of ammonia, a preferred nitrogen source for many bacteria. Also, *A. pleuropneumoniae* encodes a functional maltodextrin uptake system that is induced *in vivo*. Its role in pathogenesis remains to be established; however, in the absence of the MalT regulatory protein a stringent response is observed when *A. pleuropneumoniae* is exposed to bronchiolar lavage fluid (Lone et al. 2009a). Branched chain amino acids are also limited in the lung environment and their limitation is a cue that induces the expression of a subset of *in vivo*-induced genes via a leucine-responsive regulatory protein including *ilvI*, antisense *cps1AB*, *lrp*, and *nqr*.

An emerging theme in the pathogenesis of *A. pleuropneumoniae* is the importance of the role of genes encoding proteins involved in anaerobic metabolism. Although respiratory pathogens are generally thought of as living in comparatively oxygen-rich environments, in the tonsillar crypts, during rapid growth in acute infection, and in purulent abscesses and sequestered lesions, oxygen levels are low. To deal with these oxygen-poor environments, *A. pleuropneumoniae* uses terminal reductases that catalyze respiratory chain electron transfer to alternate terminal acceptors such as nitrate, nitrite, dimethyl sulfoxide (DMSO), trimethylamine-N-oxide (TMAO), and fumerate. Strains lacking DMSO reductase and aspartate ammonia lyase (enzymes involved in anaerobic metabolism) are avirulent (Jacobsen et al. 2005b). Also, deletion mutants of the global anaerobic transcription factor ArcA and the fumarate nitrate reduction regulator (FNR) HlyX are highly attenuated (Baltes et al. 2005; Buettner et al. 2008). Microarray and proteomics studies of a HlyX mutant reveal that in addition to regulating genes needed for adaptation to low oxygen conditions, HlyX is also involved in up-regulating the expression of a number of virulence genes including iron-regulated protein B, although the role of this highly expressed protein remains to be determined (Buettner et al. 2009).

Avoidance of Host Clearance Mechanisms

Avoiding Nonspecific Clearance. Effective host defense against bacterial infections in the lung depends on the rapid clearance of bacteria from the respiratory tract. The respiratory immune system can be divided into nonspecific (mucociliary function, complement, phagocytes) and specific lymhocyte-mediated mechanisms. The mucociliary clearance mechanism is very important in protecting the lungs from infection. Suppression of mucus production and ciliary activity by treatment with atropine and xylocaine, respectively, greatly increases the severity of disease when pigs are inoculated intrabronchially with low doses of *A. pleuropneumoniae* (Narita et al. 1995). That said, there are no data showing the effects of *A. pleuropneumoniae* infection on mucociliary activity, but it is possible that the repeat in toxin (RTX) toxins have an effect on ciliary activity.

Bacteria not cleared by normal mucociliary function may still be eliminated by the action of phagocytic cells. Both macrophages and polymorphonuclear leukocytes (PMNs) phagocytose *A. pleuropneumoniae* in the presence of convalescent pig serum (Cullen and Rycroft 1994). Following phagocytosis, PMNs can effectively kill *A. pleuropneumoniae*, whereas macrophages cannot. This difference is presumably the result of the greater bactericidal capacity of PMNs. *A. pleuropneumoniae* can survive for greater than 90 min within macrophages, during which time liberation of Apx toxins may result in lysis of these phagocytes (Cruijsen et al. 1992).

Actinobacilluspleuropneumoniae produces several factors that may contribute to its ability to survive within macrophages. High-molecular-weight surface carbohydrates present in capsule and LPS may participate in scavenging free toxic oxygen radicals (Rioux et al. 2000). The genes encoding two stress response proteins, DnaK and Trigger Factor, shown in STM studies to be essential for survival of *A. pleuropneumoniae* within the porcine respiratory tract, could also be important for survival within macrophages (Fuller et al. 2000). In addition, ammonia produced as the result of urea hydrolysis may also play a role in intracellular survival since ammonia inhibits phagosome–lysosome fusion and elevates intralysosomal pH in macrophages, resulting in depression of acid hydrolase activity (Bossé et al. 2002). Although the periplasmic location of SodC in *A. pleuropneumoniae* suggests a possible role in dismutation of superoxide radicals in phagocytic cells, *sodC* mutants are still capable of causing acute pleuropneumonia in experimental infections (Sheehan et al. 2000).

The major factors involved in the impairment of phagocytic function of both macrophages and PMNs are three RTX toxins (ApxI, ApxII, and ApxIII) produced in various combinations by the different serovars of *A. pleuropneumoniae* (table 19.3; Frey et al. 1993). At sublytic doses, these toxins impair macrophage chemotactic and phagocytic function, but stimulate macrophage and PMN oxidative metabolism (Dom et al. 1992; Tarigan et al. 1994). At high concentrations, ApxI and ApxIII are highly toxic, and ApxII is moderately toxic, for alveolar macrophages and PMNs (see "Damage to Host Tissues" section; Kamp et al. 1991; Rycroft et al. 1991; Frey 1995). However, damage to cultured alveolar macrophages by an ApxII-and ApxIII-deficient mutant of *A. pleuropneumoniae* serovar 2 indicates that other cell-associated factors may also be involved (Cullen and Rycroft 1994). The possible contribution of the recently described ApxIV toxin to macrophage and PMN damage has yet to be determined (Schaller et al. 1999).

Besides aiding phagocytes in bacterial clearance via opsonization, complement can mediate direct bacterial cytotoxicity. *A. pleuropneumoniae* is resistant, however, to the bactericidal effects of normal and immune serum, and to complement-mediated opsonophagocytosis (Rycroft and Cullen 1990; Thwaits and Kadis 1993; Ward and Inzana 1994). The main contributing factors to serum resistance by this bacterium are capsular polysaccharide (CPS) and/or LPS (Inzana et al. 1988; Rioux et al. 2000). Strains with thick adherent polysaccharide layers do not prevent activation of complement (mainly by the alternative pathway), or binding of C3 (Ward and Inzana 1994). Rather, in these organisms, binding of anti-polysaccharide antibodies occurs at a distance from the cell membrane, so that deposition of C9, a component of the membrane attack complex, is limited. Capsule-deficient strains of *A. pleuropneumoniae* serovar 5, generated by various means including allele replacement, are effectively killed in the presence of normal and immune serum, whereas encapsulated strains are not (Ward et al. 1998). In contrast, a serovar 1 capsular mutant created by transposon mutagenesis was as resistant as its parental strain to killing by normal pig serum (Rioux et al. 2000). In a transposon mutagenesis study, expression of LPS O-side chains was shown to be responsible for serum resistance in serovar 1 (Paradis et al. 1999).

Avoiding Specific Clearance Mechanisms. Since *A. pleuropneumoniae* is an extracellular pathogen, specific antibodies are critical for protection against pleuropneumonia. The possible role of cell-mediated immune response (CMIR) in protection against disease is not fully understood but there is a positive correlation among delayed-type hypersensitivity response (DTH, a measure of CMIR) to Apx toxins, increased antibody response to Apx toxins, and protection induced by low-dose infection (Furesz et al. 1997). DTH is essentially a measure of CD4+ T cell activity, and an increase in the CD4:CD8 ratio correlates with protection against pleuropneumonia (Appleyard et al. 2002). As passive transfer of serum antibodies appears to be sufficient for protection against pleuropneumonia, a CMIR does not appear to be essential (Bossé et al. 1992).

Antibodies against the Apx toxins, capsule, LPS, and certain OPMs and lipoproteins all appear to contribute to protection (Inzana et al. 1988; Devenish et al. 1990; Rossi-Campos et al. 1992; Gerlach et al. 1993; Rioux et al. 1998). In particular, anti-Apx toxin antibodies correlate strongly with (Cruijsen et al. 1995; Furesz et al. 1997), and are sufficient for (Devenish et al., 1990; Haga et al. 1997), complete protection. However, the failure of Apx-enriched bacterin and subunit vaccines to induce complete protection (Ramjeet et al. 2008) indicates that there may be differences in the level of protection obtained depending on the method of prepara-

tion of the purified toxin, and the neutralizing titer produced following vaccination, or other factors.

Both pulmonary and systemic antibodies specific for various antigens can be detected following aerosol exposure of pigs to *A. pleuropneumoniae* (Hensel et al. 1995). However, it appears that IgG, and in particular IgGl-anti-Apx toxin antibodies, are the most important in conferring protection (Furesz et al. 1998). A 101-kDa protease and a 47-kDa protease able to degrade IgG and IgA have been described, but to date there are no data to indicate that either one is able to interfere with opsonophagocytosis (García González et al. 2004).

Damage to Host Tissues. In susceptible animals, *A. pleuropneumoniae* can cause damage directly and tissue destruction can be caused indirectly via an uncontrolled pulmonary inflammatory response. Many, but not all of the pathological changes of porcine pleuropneumonia can be attributed to the Apx toxins, which have a direct cytotoxic effect on various cell types and indirectly stimulate the release of inflammatory mediators from activated phagocytes (Frey 1995). Damage to endothelial cells by Apx toxins and direct activation of factor XII by LPS initiate the coagulation, fibrinolysis, and kinin systems. Activation of the coagulation pathway results in platelet activation and the formation of microthrombi, localized ischemia, and subsequent necrosis, which are characteristic of acute porcine pleuropneumonia (Bossé et al. 2002). Activation of alveolar and intravascular macrophages, largely through Apx toxins and LPS, leads to the release of toxic oxygen metabolites (including superoxide anion, hydrogen peroxide, and hydroxyl radical), as well as proteolytic enzymes and various cytokines (Pabst 1996). Infection of pigs with *A. pleuropneumoniae* leads to rapid local production of the pro-inflammatory mediators and there is evidence for the expression of interleukin (IL) -1α, IL-1β, IL-6, IL-8, IL10, IL12p35, TNF-α, and INFα. In addition, a widely disseminated acute phase protein response following *A. pleuropneumoniae* infection has been demonstrated (Hedegaard et al. 2007; Skovgaard et al. 2009).

In immunologically naïve animals, successful defense against *A. pleuropneumoniae* requires a balance between an effective innate immune response without damaging levels of inflammation. Microarray studies of peripheral blood leukocytes from high-performing animals with low disease score versus low-performing animals with high disease score 24 h post infection revealed that 92 genes were up-regulated and four genes were down-regulated (Moser et al. 2008). Most of the differentially expressed genes found in this study are involved in innate immune response pathways. It is noteworthy that the pattern recognition molecule TLR4, the proinflammatory cytokine IL-18, and the two modulators CASP1 and 4, the macrophage inflammatory protein 1 receptor CCR1, and the interferon-γ inducible transcription factor STAT1 were expressed at higher levels in high-performing animals. Also, in this group of animals, two endogenous complement inhibitors, C1 inhibitor and apolipoprotein R precursor (C4b-binding protein), that could be involved in the attenuation of an uncontrolled inflammatory process were also up-regulated. In this study and others, there appears to be considerable animal-to-animal variation in susceptibility. Also, Benga et al. (2009) have demonstrated that there are clear differences in breed susceptibility with the Hampshire line being least susceptible while German Landrace and Piétrain breeding lines were much more likely to have severe disease following infection with *A. pleuropneumoniae*.

Although many of the pathological consequences of *A. pleuropneumoniae* infection have been attributed to LPS, very large doses of purified LPS (40–100 mg) are required to induce lesions similar to those found in naturally infected pigs (Fenwick et al. 1986). Moreover, pigs infected with a serovar 1 mutant lacking ApxI and ApxII but with normal LPS do not develop clinical disease or significant lung lesions, suggesting that the contribution of LPS to lesion development may be minimal in the absence of Apx toxins (Tascon et al. 1994). Recent studies by Ramjeet et al. (2008) demonstrated that the GalNAc-GalII-GalI region of the LPS outer core can interact with both ApxI and II, possibly via the glycine-rich Ca^{2+} domains. This interaction leads to greater hemolytic and cytotoxic activities, which they suggest might be due to stabilization of the toxins or by reducing their tendency to form aggregates.

Four RTX (repeats in toxin) toxins have been described in *A. pleuropneumoniae* (table 19.3). The ApxI and III toxins are encoded by typical RTX operons where a C gene encodes an acyl-transferase that post-transcriptionally activates the protoxin encoded by the A gene; linked B and D genes encode a type I transport system that secretes the

activated toxin. There are no transport genes linked to the *apxIICA* genes, but rather, the ApxII toxin is transported by BD proteins encoded by an incomplete *apxI* operon. Unlike the other RTX toxins of *A. pleuropneumoniae,* there are no known modification or transport genes linked to the ApxIVA structural gene and the role of the co-transcribed upstream ORF is unknown (Schaller et al. 1999). The Apx toxins are typical RTX toxins with an N-terminal hydrophobic region, glycine-rich Ca^{2+} nonapetide repeats, and a C-terminal secretion signal sequence. The ApxI toxin is strongly hemolytic and cytotoxic while the ApxII toxin is weakly hemolytic and moderately cytotoxic. At low concentrations, ApxI induces apoptosis in porcine alveolar macrophages in a caspase 3-dependent fashion (Chien et al. 2009). The ApxIII toxin lacks hemolytic activity, but is strongly cytotoxic against porcine leukocytes; porcine CD18 is necessary to mediate ApxIIIA toxin-induced leukolysis (Vanden Bergh et al. 2009). Based on the presence of antibodies in convalescent sera, there is good evidence for the expression of ApxIV *in vivo*. Under standard culture conditions, ApxIV is not produced, but in the presence of concentrated bronchoalveolar fluid, the *apxIV* gene is expressed (Lone et al. 2009b). *E. coli* expressing cloned ApxIVA along with an associated upstream ORF has weak hemolytic and cohemolytic (CAMP) activity (Schaller et al. 1999).

The critical role of Apx toxins in the development of clinical disease and tissue damage has been confirmed using recombinant toxins (rApxl, rApxII, and rApxIII; Kamp et al. 1997). Intrabronchial inoculation of pigs with either rApxl or rApxIII results in severe clinical disease and lesions indistinguishable from those seen in acutely infected animals. In contrast, inoculation with rApxII, either alone or in combination with culture filtrate depleted of Apx toxins, results in few or no clinical signs of disease and only mild lung lesions. Although ApxII appears to contribute only minimally to lesion formation, serovar 7 strains (which produce only ApxII) are capable of causing severe disease with typical lung lesions (Kamp et al. 1997). Mutants of serovar 1 and 5 strains, devoid of ApxI but still producing ApxII, also cause typical severe clinical disease and lung lesions (Tascon et al. 1994; Reimer et al. 1995). These results suggest that there may be other cell-associated toxin(s) or virulence determinant(s) contributing to the severe pulmonary lesions caused by these strains. The precise role that ApxIV plays in pathogenesis of *A. pleuropneumoniae* also remains to be elucidated, however, Liu et al. (2009) have demonstrated that ApxIVA is needed for full virulence, at least in an *apxIIC* minus background.

Apart from producing Apx toxins and LPS, *A. pleuropneumoniae* also secretes a 24-kDa protease that can degrade immnoglobulins and a 101-kDa zinc metalloprotease that is autocatalytic and forms >200-kDa complexes in culture medium (García González et al. 2004). Like other bacterial proteases, these proteases may also degrade immunoglobulins or may be involved in inactivating complement components or potentiating inflammatory processes, but their possible contribution to development of pathology has yet to be investigated. A role for urease in the pathogenesis of *A. pleuropneumoniae* has also been suggested. In addition to providing a readily available nitrogen source, an important role for urease may be in the impairment of the local immune response (Baltes et al., 2001; Bossé et al. 2002).

Although many virulence factors of *A. pleuropneumoniae* and a number of regulatory proteins have been described, much more needs to be learned about the orchestration of gene expression before a complete picture of pathogenesis will emerge. It is likely that at different stages in the infection process cell surface molecules, especially capsule and proteins adhesions, will be differentially expressed. In addition, toxin production and different metabolic pathways are probably carefully modulated in response to the various host environments.

Prevention and Control

Good management practices such as controlling air quality and temperature, avoiding overcrowding, and maintaining a strict "all-in all-out" production with thorough cleaning between groups are essential to control *A. pleuropneumoniae*. Obtaining pigs only from herds with similar or higher health status is important to control. Depopulation and repopulation and medicated early weaning schemes have also been used to eliminate the organism. The success of many of these management procedures is greatly facilitated by the use of sensitive diagnostic tests (Gottschalk and Taylor 2006).

In the event of infection, treatment of pigs with acute pleuropneumonia requires direct injection of a highly efficacious antibiotic (ideally selected after antibiotic susceptibility testing of the isolated strain). However, this is labor-intensive, time-

consuming, and expensive, and can be of limited use due to the rapid progression of the disease. Traditionally, penicillins have been used to treat *A. pleuropneumoniae*, but in many countries resistance to this group of drugs and to tetracycline, gentamicin, and tiamulin has become common (Aarestrup et al. 2008).

Evidence from the field and experimental studies indicate that infection with one serovar of *A. pleuropneumoniae* provides complete protection against subsequent infection with the homologous serovar, and at least partial protection against heterologous infection (Haesebrouck et al. 1996). This suggests that vaccination might be a feasible alternative to antibiotic treatment. Although many approaches have been tried (bacterin, recombinant subunit, and live attenuated), "a safe vaccine that offers complete protection has not yet reached the market" (Ramjeet et al. 2008). Bacterins, once widely used, do not provide good cross-protection and lack key extracellular factors such as RTX toxins; genetically inactivated ghost vaccines appear to be a more effective form of killed whole vaccine, but this approach has not been commercialized (Huter et al. 2000). Most of the currently used commercial vaccines contain combinations of recombinant toxins together with iron binding or other OMPs. Although these products have a number of advantages over the first generation bacterin vaccines, they still do not provide complete protection (Ramjeet et al. 2008). Many live attenuated vaccines (LAVs) for *A. pleuropneumoniae* have been described, and one capsule minus mutant has been licensed, but concerns regarding reversion and the lack of persistence of some metabolic mutants have limited the use of LAVs. Recently, a sixfold mutant, which is able to persist in the respiratory tract and provide heterologous protection, has been described, but even with the multiple mutations some clinical signs are still seen (Maas et al. 2006). An experimental DNA vaccine was described for the first time by Chiang et al. in 2009, but more work will be needed before such a vaccine could be commercialized.

ACTINOBACILLUS LIGNIERESII

Epidemiology

Actinobacillus lignieresii is a common resident of the oral cavity and pharynx of healthy sheep and cattle (Andrews et al. 2004), although in some early reports, isolates identified as *A. lignieresii* were misclassified (Bisgaard et al. 1986). It is the causative agent of wooden tongue, a chronic disease in cattle. It can be confused with neoplasia of the skin or lymph nodes and with actinomycosis (caused by *Actinomyces bovis* or *Staphylococcus* spp.) so culture is required for definitive diagnosis. The organism has a worldwide geographic distribution but clinical cases are generally sporadic (Campbell et al. 1975). Subclinical cases may be overlooked, so its distribution and importance may be underestimated. Studies by Christiensen et al. (2002b) showed that equine strains of "*A. lignieresii*" were genetically most closely related to Bisgard taxon 11 and *A. equuli* subsp. *haemolyticus.* Since there are no distinguishing phenotypic characteristics that allow them to be separated from *A. lignieresii,* they have been designated *Actinobacillus* genomospecies 1. Six serotypes of *A. lignieresii* have been described and autoagglinating strains are common (Nakazawa et al. 1979). There have been no reports of the use of serotyping in epidemiological studies, but such a study would be of interest as there is evidence that some strains of *A. lignieresii* are more virulent than others (Rycroft and Garside 2000).

Transmission and Disease

Although there have been no systematic studies to identify the source of infection, it is likely due to resident *A. lignieresii* from either the rumen or oropharynx. The disease, which progresses very slowly, is normally limited to the soft tissues of the head and neck, particularly the tongue (Milne et al. 2001). Damage to mucous membranes predisposes animals to infection. Typically, multiple abscesses occur on the head and under the jaw and throat region. Small, hard, pus-filled ulcers may be present on the tongue. As the disease progresses, the throat region becomes involved and the animal cannot retract its tongue. The lymphatic glands of the head and neck are often involved as well, and lesions may be present throughout the body. The lesions in the mouth can eventually lead to starvation, or pressure on the trachea may lead to asphyxiation. Abscesses around the entrance to the rumen may cause chronic bloating and other digestive disturbances. Although animals mount a detectable humoral immune response to *A. lignieresii,* antibodies do not seem to be protective (Phillips 1965).

Virulence Factors and Host Response

The virulence factors of *A. lignieresii* are unknown. Despite the fact that *A. lignieresii* and *A. pleuropneumoniae* share almost 70% DNA–DNA homology (Borr et al. 1991), the usual cutoff for classification of organisms of the same genus, and it is not possible to differentiate between these two species by a variety of molecular and phenotypic methods, the diseases caused by these species, and presumably their virulence factors, are very different. *A. lignieresii* carries homologs of the *A. pleuropneumoniae apxICABD* genes, but there are no identifiable promoter sequences upstream of this operon and the ApxI toxin is not produced (Schaller et al. 2000). *A. lignieresii* has also been shown to carry homologs of *A. pleuropneumoniae* serovar 7 and 1 *cps* genes, but not those of serovar 2, 5, 6, or 12 (Jessing et al. 2003; Angen et al. 2008). As demonstrated by positive reactions in co-agglutation tests, there appears to be a functional *cps* gene cluster in the serovar 7 gene positive strains, but in the study by Angen et al. (2008), the strain with the amplicon identical to that of the *A. pleuropneumoniae* serovar 1 *cps* gene was untypable. An LPS O-antigen that shares epitopes with *A. pleuropneumoniae* serovar 7 has also been described in *A. lignieresii* (Lebrun et al. 1999). Most likely, cell surface polysaccharides play an important role in the persistence of *A. lignieresii* in the host, but much more work is needed before their role in pathogenesis can be understood. In an early study, Phillips (1965) showed that agglutinating antibodies against heat-stable *A. lignieresii* antigens are common in apparently healthy adult animals and elevated in diseased animals. Antibody levels in young animals are typically low and do not reach adult levels until approximately 1 year of age. Given the granulomatous nature of the lesions associated with *A. lignieresii* disease, it is likely that the cellular immune response is involved in the disease, but its precise role in host protection and pathogenesis has not been described.

Prevention and Treatment

Because of its limited occurrence, there are no commercially available vaccines for *A. lignieresii* and no recent reports of any systematic study of antibiotic resistance. However, in the absence of acquired antibiotic resistance, it is likely that antibiotics used to treat other *Actinobacillus* spp. would be effective against *A. lignieresii*, but the duration of therapy required may preclude the use of more expensive products (Milne et al. 2001). Iodine solutions such as those used to treat actinomycosis are also reported to be effective in the treatment of *A. lignieresii* (Campbell et al. 1975). In the case of very valuable animals, surgery may also be an option (Boileau et al. 2009).

ACTINOBACILLUS EQUULI

Epidemiology

Actinobacillus equuli has been associated with sporadic cases of sleepy foal disease, a rare but often fatal neonatal septicemia in foals that is found throughout the world (Rycroft and Garside 2000). It is very occasionally thought to be the causative agent of disease in other species (Ramos-Vara et al. 2008). Abortion, orchitis, and peritonitis in adult horses have also been reported, but these findings are rare and, in the case of mare reproductive loss syndrome, cofactors have been implicated (Reed et al. 2003; Pusterla et al. 2008; Sebastian et al. 2008). In 2002, Christensen et al. (2002a) proposed a new classification scheme for equine isolates of *A. equuli,* which had previously been variously reported as *A. equuli,* variants of *A. equuli, Actinobacillus suis, A. suis*-like, or Bisgaard taxon 11. Based on 16S rRNA and DNA–DNA hybridization studies, they recommended that these organisms be classified as *A. equuli* subsp. *equuli* subsp. nov. and *A. equuli* subsp. *haemolyticus* subsp. nov. Based on ribotyping and biotyping, *A. equuli* are quite heterogeneous. These findings are consistent with early reports by Kim et al. (1976) who reported more than 28 heat-stable antigenic groups. In light of later genetic studies, previous biotyping schemes (e.g., ability to ferment arabinose) that had been used to classify *A. equuli* may not be that useful (Sternberg and Brandstrom 1999).

Transmission and Disease

Actinobacillus equuli is a common resident of the oral cavity of adult horses although it is sometimes difficult to culture (Phillips 1984; Sternberg and Brandstrom 1999). It has also been recovered from feces of healthy horses. *A. equuli* can be transmitted from the mare to the foal via the oral, respiratory, or umbilical route at or following birth. It has also been suggested that the organism can be transmitted *in utero.* Mares may carry multiple strains of *A. equuli* and it is likely that strains of *A. equuli* subsp.

haemolyticus are more likely to cause disease than *A. equuli* subsp. *equuli,* but this remains to be demonstrated (Donahue et al. 2006; Holyoak et al. 2007). In young foals, *A. equuli* can cause an acute septicemia also known as sleepy foal disease (Rycroft and Garside 2000). In animals less than 1 month of age, *A. equuli* suppurative nephritis, arthritis, pneumonia, pleuritis, or enteritis is frequently seen in association with septicemia. Mares can produce antibodies to resident *A. equuli* and it is these antibodies, passed to foals through colostrum, that are apparently associated with protection (Rycroft et al. 1998; Berthoud et al. 2004).

Pathogenesis

Virtually nothing is known about the virulence mechanisms of *A. equuli.* Like *A. pleuropneumoniae* and *A. suis,* hemolytic strains of *A. equuli* produce an RTX toxin (Berthoud et al. 2002; Kuhnert et al. 2003a, 2003b). This toxin is encoded by the *aqxA* gene, which lies within a typical RTX cluster (*aqxCABD*). It has a predicted molecular mass of 110 kDa and has nine characteristic glycine-rich nonapeptide repeats. The AqxA protein most closely resembles the LktA protein of *Mannheimia haemolytica,* whereas the B and D proteins have greater than 90% homology with the ApxIB and D proteins of *A. pleuropneumoniae.* The *aqxCABD* operon is present in all hemolytic strains of *A. equuli,* but absent from nonhemolytic *A. equuli* (Berthoud et al. 2002). It is tempting to speculate that the AqxA toxin may have a role in virulence, but this remains to be tested. However, as both hemolytic and nonhemolytic strains can be isolated in the same foal at different sites in the body, it is also possible that the immune status of the foal plays the determining role in the outcome of infection (Rycroft et al. 1998; Berthoud et al. 2004).

Prevention and Treatment

Although there is some indirect evidence that *A. equuli* subsp. *haemolyticus* is more likely to cause disease than *A. equuli* subsp. *equuli* (Donahue et al. 2006; Holyoak et al. 2007) it is generally thought that all *A. equuli* have pathogenic potential. Consistent with this, Sternberg and Brandstrom (1999) reported that it was not possible to distinguish between clinical isolates and normal flora by either ribotyping or phenotyping; however, hemolytic activity was not evaluated in this study. This finding, together with the fact that there seems to be a large number of serotypes, suggests that the prospects for development of a vaccine are not bright unless antibodies to the Aqx toxin or other common virulence factor(s) can provide heterologous protection. Although antibiotic resistance is reported to be on the rise in bacterial pathogens of horses (Fletcher et al. 2004), there have been no recent reports of systematic antibiotic sensitivity testing of *A. equuli* isolates; cefquinome is reported to exhibit very low minimal inhibitory concentrations (MICs) against *A. equuli* (MIC_{90} of 0.016; Thomas et al. 2006).

ACTINOBACILLUS SUIS

Epidemiology

Actinobacillus suis is a common commensal of swine, but under conditions that are poorly understood, it can cause pneumonia, septicemia, and a variety of other diseases including abortion (MacInnes and Desrosiers, 1999; Mauch and Bilkei, 2004; MacInnes et al. 2008). It can be cultured from the nasal passages and more readily from the tonsils of swine of all ages. Since its original description in 1962, sporadic cases of infection with *A. suis* or *A. suis*-like organisms have been reported in a variety of birds and mammals. It is likely that many of the early reports of isolates from non-porcine sources were probably not *A. suis sensu stricto,* but there is evidence that at least some strains of *A. suis* can infect mammals other than swine (Christensen et al. 2002a; Jeannotte et al. 2002). *A. suis* was traditionally thought of as the causative agent of septicemia in very young pigs. However, beginning in the early l990s, there have been increasing numbers of reports of severe outbreaks of disease due to *A. suis* in Canada, the United States, and Australia in pigs of all ages (MacInnes and Desrosiers 1999; Wilson and McOrist 2000; Taylor 2006).

Early studies of the population structure of *A. suis* revealed very little heterogeneity among *A. suis* isolates cultured from healthy and diseased swine (Bada et al. 1996; van Ostaaijen et al. 1997). Later studies by Slavic et al. (2000a) revealed that there are at least two O types of *A. suis* and that O2 isolates were more likely to be associated with severe disease. However, they postulated that it was the CPS rather than the LPS that may have been the more important determinant of pathogenesis (Slavic et al. 2000a, 2000b). Chemical characterization of the surface polysaccharides of representative strains revealed that the O1 antigen is (1-6)-β-D-glucan and

the O2 antigen is a (Glc, Gal2, GlcNac) branched tetrasaccharide (Monteiro et al. 2000; Rullo et al. 2006). Several different capsular types have also been identified. The Kl capsule is (l-6)-β-D-glucan while the K2 and K3 capsules contain sialic acid, but their precise structure remains to be determined. Very recently, a short polysaccharide antigen has also been identified in both K1 and K2 strains. Attempts to develop a serotyping system for *A. suis* have been thwarted by the presence of this (or some other) common antigen and the absence of sequence data for the CPS biosynthesis genes.

Transmission and Disease

Actinobacillus suis rapidly loses viability in pathological samples and culture media, and although there have been no systematic studies, it is unlikely that the organism survives in the environment for any appreciable time (Taylor 2006). *A. suis* infection can occur via the aerosol route or by close contact. The organism may also gain entry through breaks in the skin (e.g., during castration). Once the organism has entered the bloodstream, it spreads rapidly throughout the body (Taylor 2006). In conventionally reared swine, *A. suis* is a commensal organism in the tonsils and upper respiratory tract, and an opportunistic pathogen. In high health status herds, *A. suis* may not be present and animals of all ages in these herds may succumb to severe *A. suis* disease if the infection is introduced. Once herd immunity has been established, however, the number of disease outbreaks decreases (Taylor 2006). In neonates and sucking pigs, *A. suis* can cause an acute and rapidly fatal septicemia where death occurs within 15 h. Affected animals may show signs of cyanosis, petechial hemorrhage, fever, respiratory distress, neurological disturbances, and arthritis. In slightly older animals, the disease is less severe and may be characterized by fever, anorexia, and persistent cough. Although mortality is much lower, these animals tend to be poor doers. In mature animals, *A. suis* infection can be confused with erysipelas. These animals may have erythematous skin lesions, fever, and inappetence; abortion, metritis, and meningitis have also been reported in sows. Once in the bloodstream, *A. suis* can form microcolonies on vessel walls that lead to regions of hemorrhage and necrosis. Gross lesions are usually seen in the lungs, kidney, heart, spleen, intestines, and skin. The lungs may also be filled with a serous or serofibrinous exudate and superficially look like lungs of animals with pleuropneumonia. Occasionally, animals are seen with an acute necrotizing myocarditis that is reminiscent of mulberry heart disease (MacInnes and Desrosiers 1999).

Pathogenesis

In contrast to *A. pleuropneumoniae,* relatively little is known about the pathogenesis of *A. suis* although a mouse challenge system that might help in future investigations has been described (Ojha et al. 2007). Despite the many superficial similarities between *A. pleuropneumoniae* and *A. suis, A. suis* does not hybridize in *A. pleuropneumoniae* microarrays and shares only 50% DNA–DNA homology (Borr et al. 1991, Gouré et al. 2009). Moreover, these organisms cause distinct diseases and presumably have a number of different virulence factors. Although *A. suis* is generally thought to be less pathogenic, it seems better able to invade the bloodstream and unlike *A. pleuropneumoniae,* it can be isolated from the alimentary tract and vagina.

In signature tagged mutagenesis studies, a number of metabolic genes and several putative cell surface proteins were found to be essential for colonization (Ojha et al. 2005). One of the putative adhesins identified in these studies, an OmpA homolog, was necessary for adhesion to porcine brain microvascular endothelial cells (Ojha et al. 2010). *A. pleuropneumoniae* on the other hand is unable to bind to porcine brain microvascular endothelial cells and it is possible that differences in OmpA may at least in part, explain the differences seen in tissue tropism. The presence of an ~12-kbp “colonization island” has recently been described in GenBank (accession no. EU427462.1) that is 95% and 92% identical to a comparable region in *A. pleuropneumoniae* serovars 5 and 7, respectively. On the basis of homology with other sequences, it would appear that *A. suis* is likely to express fimbriae and form biofilms. As with *A. pleuropneumoniae,* LPS could play a role in attachment, but again, this remains to be tested.

A number of “virulence” factors associated with nutrient acquisition are also present in *A. suis.* For example, *A. suis* is strongly urease-positive and can bind porcine (but not human or bovine) transferrin using TbpA and TbpB. These proteins are virtually identical to those in *A. pleuropneumoniae* (Bahrami et al. 2003). *A. suis* also expresses a hemoglobin-binding protein, again very similar to that of *A. pleuropneumoniae* (Bahrami and Niven 2005).

The mechanism of invasion, except in the case of direct entry in the bloodstream, is another area of *A. suis* pathogenesis that is not understood. Once in the bloodstream, capsule and LPS undoubtedly play a role in the survival of the organism. Regardless of their O and K types, all *A. suis* isolates appear to be serum-resistant and, in fact, grow better in the presence of fresh serum than in serum that has been heat-inactivated (Slavic et al. 2000b). The RTX toxins, ApxI and ApxII, that are virtually identical to those expressed in *A. pleuropneumoniae,* also likely play a role in the pathogenesis of *A. suis,* but it appears that they are expressed at a lower level (van Ostaaijen et al. 1997). This lower level of expression may facilitate entry of the organism into the bloodstream via tonsillar lymphocytes, but this remains to be demonstrated. In contrast to *A. pleuropneumoniae, A. suis* lacks the *apxIV* genes (Schaller et al. 1999). *A. suis* produces a protease that can cleave pig and bovine IgG that cross-reacts with polyclonal serum against a high-molecular-mass secreted protease from *A. pleuropneumoniae*, but any role in pathogenesis has yet to be demonstrated (Negrete-Abascal et al. 2004).

Prevention and Control

As noted above, maintenance of good management practices is key to disease control (Maes et al. 2001). At present, there are no commercial vaccines for *A. suis,* but autogenous bacterins are thought to be useful (Lapointe et al. 2001; Taylor, 2006). Theoretically, *A. pleuropneumoniae* vaccines, especially those with Apx toxins and OMPs, could provide some cross-protection (MacInnes and Rosendal 1987; Devenish et al. 1989). There are no commercially available serodiagnostic tests for *A. suis*, but Lapointe et al. (2001) used an ELISA-based test on a saline extract of boiled formalinized whole cells of a field strain to measure antibody levels in response to vaccination. Isolates of *A. suis* are often resistant to antibiotics, but antibiotic therapy is often impractical, given the very sudden onset of disease. Although there have been no systematic reports of antibiotic resistance in *A. suis*, it is noteworthy that a plasmid-mediated *tetB* gene has been described (GenBank, Accession no. GQ229163.1) that is 99% identical to *tetB* in *Steptococcus suis*, *E. coli*, and a number of serovars of *Salmonella enterica* subsp. enterica.

FUTURE PROSPECTS

Our understanding of the virulence mechanisms of members of the genus *Actinobacillus* lags far behind that of many other animal pathogens, and more effective vaccines and diagnostic tests are still needed for this group. Although there is some information about the interactions between various *Actinobacillus* species and the host innate and acquired immune systems, almost nothing is known about host receptors and their cognate bacterial adhesins. More work is also needed to understand the factors that regulate the expression of virulence factors and essential metabolic pathways, especially as they apply to our understanding of the transition from harmless commensal to acute pathogen. Analysis of emerging genomics and proteomics data will no doubt be helpful in solving some of these mysteries. In the not so distant future, current serotyping and biotyping schemes may be replaced with PCR and sequencing-based methods that target cell surface antigens or specific virulence factors, and there may also be an effort to license live attenuated vaccines or more complex recombinant products.

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20
Haemophilus

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INTRODUCTION

Species within the genus *Haemophilus* are obligate inhabitants of animals and humans. The name *Haemophilus* means "blood-loving," because blood or blood factors were originally required to isolate these bacteria. In most cases, either nicotinamide adenine dinucleotide (NAD or NAD phosphate; V factor) or protoporphyrin IX or protoheme compounds such as hemin (X factor), or both, are required for growth. *Haemophilus somnus*, now reclassified as *Histophilus somni*, is one exception to this rule because it requires blood factors for growth, but not NAD or hemin per se. Another characteristic of members of the genus *Haemophilus* is host specificity. Most *Haemophilus* species establish a commensal relationship with their host, usually inhabiting the upper respiratory tract, but in some cases the genital tract. However, some species can disseminate via the bloodstream and cause pneumonia or serious systemic illness. In most cases, infections occur as a result of inadequate immunity, intercurrent or previous infection, severe stress, or a combination of factors.

Table 20.1 lists the *Haemophilus* spp. (or previously classified as *Haemophilus* spp.) of animals currently recognized, their normal hosts, and associated disease(s). Many species are also commensals or pathogens in the respiratory or genital tracts of humans, including the type species *Haemophilus influenzae*, which is a common cause of otitis media, pneumonia, meningitis, epiglotitis, and bacteremia. Although not a pathogen of animals, *H. influenzae* will be referred to because of the large volume of work with this pathogen. Another extensively examined human pathogen is *Haemophilus ducreyi*, the etiologic agent of the sexually transmitted disease chancroid. Furthermore, many *Haemophilus* spp., primarily commensal inhabitants of animals, have likely not been identified because they are rarely or never isolated from clinical specimens.

CHARACTERISTICS

Species in the genus *Haemophilus* are pleomorphic, gram-negative rods or coccobacilli. They may vary in length, sometimes forming filaments simultaneously with short coccobacilli. All species ferment carbohydrates and reduce nitrates, and are facultative anaerobes (Kilian and Biberstein 1984). Even with the addition of blood factors, complex media, such as Levinthal's, brain heart infusion, or Columbia, are required for growth. However, there is a defined medium for *H. somni* (Inzana and Corbeil 1987). Colonies are non-pigmented to gray or slightly yellow.

Many species and strains of pathogens are encapsulated. The cell membrane is typical of gram-negative bacteria. Endotoxin is a major component of the outer membrane, and is commonly referred to as lipooligosaccharide (LOS), rather than lipopolysaccharide (LPS), because of the lack of O-side chains in all species examined (Hitchcock et al. 1986). Six to eight proteins predominate in the outer membrane. However, absence of iron induces synthesis and placement in the membrane of additional proteins that bind specifically to host transferrin (Yu et al. 1992), sequestering iron for the bacterium. The specificity of the transferrin-binding proteins, the variety of adherence factors

Table 20.1. *Haemophilus* and Related Species in Animals

Host	Species	Normal habitat	Associated disease
Pigs	*Haemophilus parasuis*	URT	Polyserositis (Glässer's disease) respiratory disease, septicemia, arthritis
Poultry	*Avibacterium paragallinarum*	URT	Fowl coryza
Cats	*H. felis*	URT	Upper respiratory infection (rare)
Dogs	*Haemophilus haemoglobinophilus*	UGT	Neonatal mortality (rare)
Cattle, sheep	*Histophilus somni*	URT, GT	Cattle: thrombotic meningoencephalitis, pneumonia, reproductive failure, septicemia, myocarditis, arthritis Sheep: epididymitis, pneumonia, septicemia, meningitis, arthritis, myositis, mastitis
Horses	*Taylorella (H.) equigenitalis*	GT	Abortion and infertility

URT—upper respiratory tract; GT—genital tract.

required for colonization, and innate host immunity may account for the stringent host specificity of *Haemophilus* spp. Colonization factors include specific components on the LOS, such as phosphorylcholine (ChoP), pili, and some surface proteins. A variety of other factors are also produced, some of which may be species-specific.

HABITAT AND TRANSMISSION

Each *Haemophilus* spp. is an obligate parasite of the mucosal surfaces of a single mammalian host or closely related species. These fastidious bacteria cannot survive long periods in the environment, and colonize new hosts by direct contact with a host that has clinical disease, that is a post-clinical carrier of the bacterium, or that is an asymptomatic carrier, through contaminated fomites, or by inhalation of infectious aerosols. Such transmission may occur over relatively long distances. For example, transmission of the related host-specific bacterium *Actinobacillus pleuropneumoniae* can occur via air vents over a distance of ≥2.5 meters (Jobert et al. 2000).

Under most circumstances, *Haemophilus* spp. exist as commensals in the upper respiratory or urogenital tracts. For reasons that are not well understood, the bacteria may gain access to the lower respiratory tract or the bloodstream and invade other organ systems, such as the central nervous system. However, dissemination of these bacteria is most likely a consequence of the virulence of the pathogen and of compromised host defenses, perhaps due to stress from crowding or shipping, previous viral infection, mycoplasma, other infections, or other conditions that would suppress innate and adaptive host immunity. This may explain, in part, why the predominant pathogenic *Haemophilus* spp. occur in animals subjected to intense management practices. In addition, some species or strains are particularly well adapted to escape host defense mechanisms. This chapter will focus on the virulence factors of the three predominant *Haemophilus* (or recently reclassified) pathogens of animals (*Haemophilus parasuis*, *Avibacterium paragallinarum*, and *H. somni*), and how these factors contribute to the disease process or enable the bacteria to avoid host defense mechanisms.

HISTOPHILUS SOMNI

Overview

Haemophili that resemble *H. somni* have been isolated from cattle, sheep, bison, and bighorn sheep. The ovine isolates have been variously described as *Histophilus ovis*, *Haemophilus agni*, and *H. somnus.* Isolates characterized as *H. somnus* cause several syndromes in cattle and sheep, including thrombotic meningoencephalitis (TME), upper respiratory infection, pneumonia, bacteremia, abortion, arthritis,

myocarditis, infertility, and ovine epididymitis (Inzana 1999). Although the organism may cause any of these conditions, it is a common genital tract commensal, found especially on the prepuce of bulls and rams. *H. somnus* can be carried in the upper respiratory tract, but is isolated from disease most often from the lower respiratory tract. *H. agni* and *H. ovis* are primarily carried in the genital tract of sheep, but may cause vulvitis, low reproductive rates, mastitis, septicemia, arthritis, meningitis, pneumonia, and, in young rams, epididymitis (Cassidy et al. 1997). These bacteria have been isolated primarily in Australia and New Zealand, but also in Canada.

The taxonomic position of these species has apparently been resolved. Angen et al. (2003) proposed that *H. somnus*, *H. agni*, and *H. ovis* constitute a single species, which should be moved to the genus *Histophilus* and named *H. somni.* These conclusions are based on sequencing of the 16S rRNA and *rpoB* genes, on DNA-DNA hybridization, and on the phenotypic traits capnophilia, yellowish pigment, and indole production. The discussion of *H. somni* in this chapter is based on work with bovine isolates, but it should also apply to ovine isolates.

Host-Pathogen Interactions

The portal of entry for *H. somni* is usually the respiratory or genital tract. Interaction with the normal flora at the site of initial entry may influence the outcome of the exposure. An investigation of the effect of 30 normal flora isolates from the healthy prepuce and 40 from the nasal cavity on growth of *H. somni in vitro* showed that the number of isolates that enhance the growth of *H. somni* outnumbered isolates that inhibit growth by 4 : 1. Only a few isolates did not affect *H. somni* growth *in vitro* (Corbeil et al. 1985). Therefore, most normal flora do not inhibit the growth of *H. somni*, but inhibitory flora may act as a first line of defense. The mucociliary elevator is a second line of respiratory tract defense against *H. somni* colonization. After crossing this barrier, it must attach to epithelial cells and pass through endothelial cells to invade the bloodstream.

Histophilus somni attaches to bovine turbinate cells, vaginal epithelial cells, and endothelial cells (Sylte et al. 2001). This may be mediated by heparin-binding proteins, which interact with sulfated proteoglycans on endothelial cells (Behling-Kelly et al. 2006). Interaction of viable *H. somni* with bovine brain endothelial cells results in cytoskeletal rearrangement, significant increases in albumin flux, and reduction in transendothelial electrical resistance, which may alter the integrity of the blood-brain barrier (BBB) and increase paracellular permeability of vascular endothelial cells (Behling-Kelly et al. 2007). Subsequently, *H. somni* and its LOS can activate caspase and induce apoptosis of bovine pulmonary and brain vascular endothelial cells (Sylte et al. 2001, 2003, 2006), although treatment of endothelial cells with IL-1β diminishes LOS-mediated apoptosis of bovine pulmonary artery endothelial cells (Sylte et al. 2005). Furthermore, *H. somni* and its LOS (most likely through expression of ChoP) activate bovine platelets, which in turn can induce endothelial cell apoptosis (Kuckleburg et al. 2005). The resulting platelet activation generates a pro-inflammatory response that may contribute to vascular inflammation and endothelial cell damage characteristic of *H. somni* disease. The organism compromises not only endothelial cells but also phagocytic cells. *H. somni* also modulates bovine neutrophils (polymorphonuclear leukocyte [PMN]) and macrophage function, decreasing their bactericidal activity and inducing apoptosis (Yang et al. 1998).

Histophilus somni can modulate phagocyte function and survive intracellularly, probably through compromising and killing the phagtocytic cells. Chiang et al. (1986) showed that *H. somni* produces PMN-suppressive factors identified as purine and pyrimidine bases, as well as ribonucleotides and a ribonucleoside. Intracellular survival studies have been extended to bovine monocytes and macrophages and include inhibition of the phagocytic cell oxidative burst (Lederer et al. 1987; Sample and Czuprynski, 1991; Pfeifer et al. 1992; Gomis et al. 1997). Howard et al. (2002) demonstrated significant inhibition of superoxide anion (O_2^-) produced by bovine mammary and alveolar macrophages and PMN previously infected with live pathogenic strains of *H. somni*, and stimulated with phorbol 12-myristate 13-acetate (PMA), but not by cells infected with *Escherichia coli* or *H. influenzae*. The inhibition of O_2^- was time- and dose-dependent, required contact with, but not phagocytosis of, live *H. somni*, and isolates from the normal bovine prepuce were less capable or incapable of inhibiting O_2^- production. However, it has yet to be determined if inhibition of the oxidative burst contributes to intracellular survival.

Histophilus somni also produces histamine, probably through decarboxylation of histidine (Ruby et al. 2002). Secretion is enhanced under conditions of high CO_2 concentrations, which may approximate those in the bronchi. As a result, production of histamine, which is a mediator of the inflammatory process, may contribute to the pathogenesis of respiratory disease.

Environmental Factors in Disease

Clinical observations reveal stress as an important component in susceptibility to *H. somni* infection, especially as part of the "shipping fever" complex (or bovine respiratory disease complex). The role of viral infection in decreasing host defenses is not completely defined, although production of IFN-γ following viral infection may enhance susceptibility to secondary bacterial infection (Sun and Metzger, 2008). Nasal infection with bovine respiratory syncytial virus (BRSV) 6 days before intrabronchial challenge with *H. somni* results in more severe disease than with either pathogen alone (Gershwin et al. 2005). Similarly, healthy calves pre-challenged with bovine herpes virus-1 are more susceptible to *Mannheimia haemolytica*-induced pneumonia and systemic disease (Potter et al. 1999). Crowding of cattle probably increases animal:animal contact and contributes to stress. Anecdotally, cold weather predisposes cattle to *H. somni*-induced disease, especially sudden decreases in environmental temperatures. *H. somni* infection often results in bacteremia with localization in sites of predilection (brain, placenta, joints, myocardium, and lung). In each case, PMN infiltration at the site of infection is characteristic. The resulting vasculitis and thrombosis, hallmarks of *H. somni* infection, involve small- or medium-sized arteries as well as venules, and may be accompanied by hemorrhage and edema. Vasculitis and thrombosis occur in most tissues infected by *H. somni*, but the duration of infection probably determines whether it is detected clinically. The route of experimental infection may determine whether this "hallmark" is detected. For example, vasculitis is typical in experimental thromboembolic meningoencephalitis (TME) induced by intravenous inoculation. However, *H. somni* administered intrathecally results in purulent meningitis but not vasculitis (Lees et al. 1994). In experimental *H. somni* pneumonia, degeneration of alveolar macrophages is a consistent finding 24 h after intrabronchial inoculation (Gogolewski et al. 1987), suggesting that *H. somni* may destroy macrophages before they kill the pathogen. In the above study, *H. somni* was found either free in alveoli or associated with degenerate alveolar macrophages.

Comparative genomics of *Histophilus somni*

Comparative analysis of the genomes of *H. somni* pneumonia isolate strain 2336 and strain 129Pt from the healthy prepuce has provided insight into their evolutionary relationships and identified similarities and differences in genome architecture and arrangement (S. Siddaramappa, unpublished data). Strain 2336 has a greater number of unique genes (16%) than strain 129Pt (8%; Challacombe et al. 2008). The majority of genes unique to strain 129Pt are not functionally characterized (annotated as hypothetical and conserved hypothetical), while others encode phage-related functions (fig. 20.1). Strain 2336-unique genes that may contribute to virulence include LOS biosynthesis genes, autotransporter adhesins, 11 homologs of filamentous hemagglutinins, (including an immunoglobulin-binding protein [IgBP]), as well as restriction modification (RM) systems (S. Siddaramappa Ph.D. dissertation, Virginia Tech, 2007). Seven prophage regions, distributed randomly in the chromosome, appear to have enriched the metabolic and pathogenic potential of strain 2336. The presence of a putative subtilase in strain 2336, which lacks homologs in other members of the *Pasteurellaceae*, and RM enzymes in both strains, are examples of bacteriophage-mediated horizontal gene transfer. Therefore, comparison of the chromosomes of strains 129Pt and 2336 supports the proposition that bacteriophages have played a major role in creating genomic diversity and phenotypic variability among different strains.

Virulence Factors

Virulence in *H. somni* involves colonization, invasion, host cell damage, and evasion of the host response. Factors that enable the bacteria to invade host tissues and avoid clearance by host defenses are not shared by all *H. somni* strains.

LOS

LOS of *H. somni* is similar in structure and function to that of *H. influenzae* and *Neisseria gonorrheae.* It is microheterogeneous and lacks the O-side chains of LPS. The lipid A component has endotoxic activity, and is similar in composition to that of *E. coil* (Inzana et al. 1988). The inner and outer core

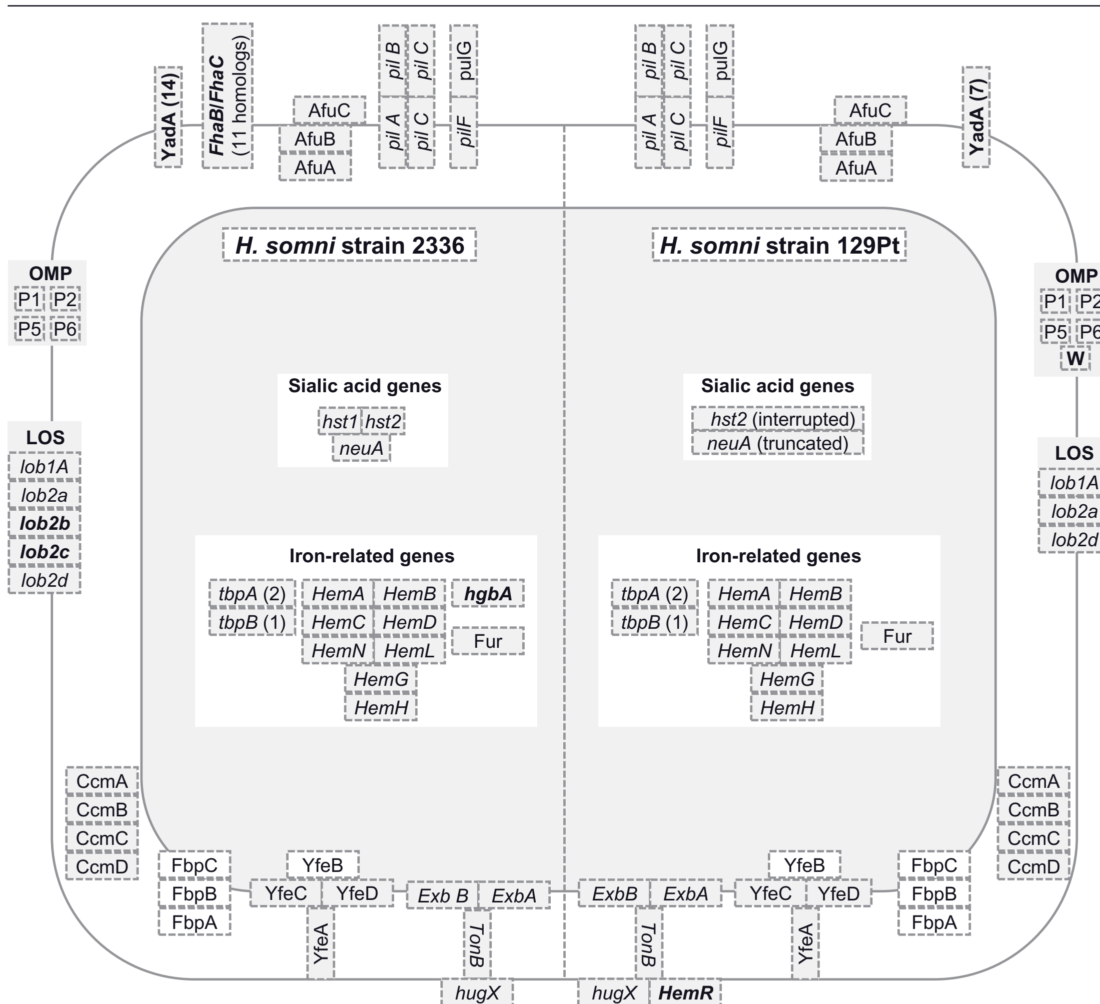

Figure 20.1. Comparison of putative virulence factors of *H. somni* strains 2336 and 129Pt.

oligosaccharide is very similar in structure to that of *N. gonorrheae*, and contains lacto-N-neotetraose or lacto-N-tetraose (Cox et al. 1998) oligosaccharides also present on mammalian cell glycosphingolipids. Thus, similarity to host antigens may cause the oligosaccharide to be poorly immunogenic. In addition, the outer core oligosaccharide can undergo a high rate of random structural and antigenic phase variation. However, phase variation occurs predominantly in isolates recovered from disease and never or seldom in serum-sensitive preputial isolates from healthy animals (Inzana et al. 1992, 1997).

The potential for extensive antigenic heterogeneity in *H. somni* has in part to do with LOS phase variation. Phase variation in *H. influenzae* and *H. somni* is due, at least in part, to microsatellites of repeating DNA motifs in the open reading frame (ORF; Weiser et al. 1989, Inzana et al. 1992). Some genes have microsatellites of a repeating DNA motif (e.g., 5′-CAAT-3′) immediately downstream from the start codon(s) or anywhere in the ORF. During replication, slipped-strand mispairing may result in the loss or gain of one or more repeats. This variation can place the ORF in or out of frame, resulting in a translational switch. We have identified five genes with homology to glycosyltransferases in *H. somni.* Two genes, *lob1* and *lob2A*, are putative galactose and N-acetylglucosamine transferases, respectively

(McQuiston et al. 2000; Wu et al. 2000). *lob1* contains microsatellites of 5′-CAAT-3′ immediately downstream of the start codons, but has no homology to any of the *H. influenzae lic* genes, which also contain variable repeats of 5′-CAAT-3′. In contrast, *lob2A* contains microsatellites of 5′-GA-3′ near the 3′ end of the ORF. In addition, *lob2BCD* occurs immediately downstream and appears to form a locus with *lob2A*. *lob2B* is almost identical in sequence to *lob2A* and also contains repetitive sequences of 5′-GA-3′ in the same location. *lob2C* lacks any repetitive DNA sequences, but *lob2D* contains microsatellites of 5′-CAGT-3′ in the middle of the ORF (Duncan et al. 2002). Mutagenesis of *lob2A* resulted in an *H. somni* mutant with a lower rate of phase variation and diminished virulence in a mouse model. *H. somni* 129Pt contains *lob1* and *lob2D*, but lacks the full sequences of *lob2ABC* (Challacombe et al. 2007). Sequence analysis revealed amino acid differences in *lob1* between strains 2336 and 129Pt, and extensive analysis of *lob2D* in multiple colonies of 129Pt showed that the number of repeats in the gene would always correlate with the gene being in the off phase, thus explaining why phase variation was not detected in 129Pt unless it was complemented with *lob1* from strain 2336 (McQuiston et al. 2000).

The LOS of *H. somni* contains ChoP, whose expression varies due to a variable number of tandem repeats of the tetranucleotide unit 5′-AACC-3 in the ORF of *lic1A*, one of the five genes responsible for ChoP expression (Howard et al. 2000). Expression of ChoP is associated with colonization of the bovine respiratory tract, which appears to be moderated through binding to the platelet activating factor receptor (PAF-R) found on platelets, and epithelial and endothelial cells. In contrast, when ChoP phase varies off, bacteria with this phenotype predominate in systemic sites (S. Elswaifi, Ph.D. dissertation, Virginia Tech). *H. influenzae* ChoP binds to C-reactive protein, resulting in complement activation and bactericidal activity (Weiser et al. 1998). Therefore, phase variation of ChoP may enable colonization of the upper respiratory tract, followed by systemic invasion of host tissues. However, some isolates from the normal bovine prepuce do not phase vary and do not contain detectable ChoP (Inzana et al. 1997; Howard et al. 2000). Therefore, adherence of *H. somni* to host cells is likely to depend on multiple factors, including various bacterial proteins and host cell receptors.

H. influenzae, *H. ducreyi*, and apparently *H. somni* catabolize N-acetyl-5-neuraminic (sialic) acid (Neu5Ac), and can thereby incorporate Neu5Ac into their LOS through metabolic pathways (Vimr et al. 2000; Schilling et al. 2001; Inzana et al. 2002). Genes in the *siaPT* and *nan* operons, and *siaR*, respectively, encode proteins required for transport, catabolism, and regulation of Neu5Ac in *H. influenzae* (Johnston and Apicella 2008). A screen of *H. somni* genomes via BLASTx identified ORFs that would encode enyzmes with homology to each of these *H. influenzae* metabolic Neu5Ac genes. In addition, four ORFs with homology to *H. influenzae* sialyltransferases *siaA*, *lsgB*, *lic3A*, and *lic3B* have been identified, as well as genes with homology to a CMP-sialylsynthetase (*siaB*). Therefore, *H. somni* appears to be capable of metabolizing and decorating its cell surface with Neu5Ac in a manner similar to that seen in *H. influenzae*. Expression and thin layer chromatography of these genes in *E. coli* indicated that the LsgB homolog sialyltransferase preferentially sialylates N-acetyl-lactosamine (Gal-[1-4]-β-N-acetyl-glucosamine), whereas the SiaA homolog preferentially sialylated lacto-N-biose (Gal-[1-3]-β-N-acetyl-glucosamine; M. Howard, PhD dissertation, Virginia Tech, 2005). In strain 2336 the terminal β-gal-β-N-acetyl-glucosamine can phase vary between a 1-4 linkage and a 1-3 linkage. Thus, two sialyltransferases with distinct specificities can accommodate sialylation of the LOS, regardless of the structure of the terminal linkage. However, Neu5Ac has not been detected on the LOS of serum-sensitive isolates tested from the normal prepuce. Two sialyltransferase homologs (LsgB and SiaA) and the sialylsynthetase are either missing from or truncated in the genome of preputial isolate 129Pt, and are, therefore, not functional (Challacombe et al. 2007). The addition of Neu5Ac onto the LOS inhibits antibody binding to *H. somni* and enhances serum resistance (Vimr et al. 2000; Schilling et al. 2001; Inzana et al. 2002). Sialylated LOS also binds more complement Factor H than non-sialylated LOS, resulting in less activation and consumption of complement. Furthermore, sialylation of *H. somni* inhibits phagocytosis and intracellular killing of the bacteria (R. Balyan, MS Thesis, Virginia Tech, 2007). Therefore, *H. somni* LOS is a multifactorial virulence component: the lipid A component has endotoxic activity, while the oligosaccharide may promote colonization and

resistance to host defenses through phase variation, and expression of ChoP, Neu5Ac, and possibly other constituents.

Biofilm Formation

In Vitro. Bacterial biofilms are aggregations of bacteria that live in a highly structured and organized community. *H. somni* readily forms biofilm communities *in vitro* and *in vivo* (Sandal et al. 2007; Sandal et al. 2009) (fig. 20.2). An interesting feature of *H. somni* is the substantial difference in the amount and architecture of biofilm formed by strain 2336, isolated from bovine pneumonia, compared with commensal preputial strain 129Pt. Strain 2336 produces a mature biofilm that consists of thick, homogenous mound-shaped microcolonies encased in an amorphous extracellular matrix with profound water channels (fig. 20.2A4). In contrast, strain 129Pt forms a biofilm of cell clusters that are tower-shaped or distinct filamentous structures intertwined with each other by strands of extracellular matrix (Sandal et al. 2007; fig. 20.2A2). As in many bacterial species, biofilm formation in *H. somni* progresses through multiple developmental stages (Davey and O'Toole 2000; Davies and Geesy 1995; Sauer et al. 2002; Schembri et al. 2003) and displays a distinct, phenotypic life cycle that includes stages of attachment, growth, maturation, and detachment (figs. 20.2A1 and 20.A3).

The differences in biofilm architecture by the two strains may correlate with virulence, since strain 2336 forms a significantly larger amount of biofilm than commensal strain 129Pt. Since *H. somni* is an obligate inhabitant of bovines, the formation of such a prominent biofilm by strain 2336 suggests that this structure may be important for

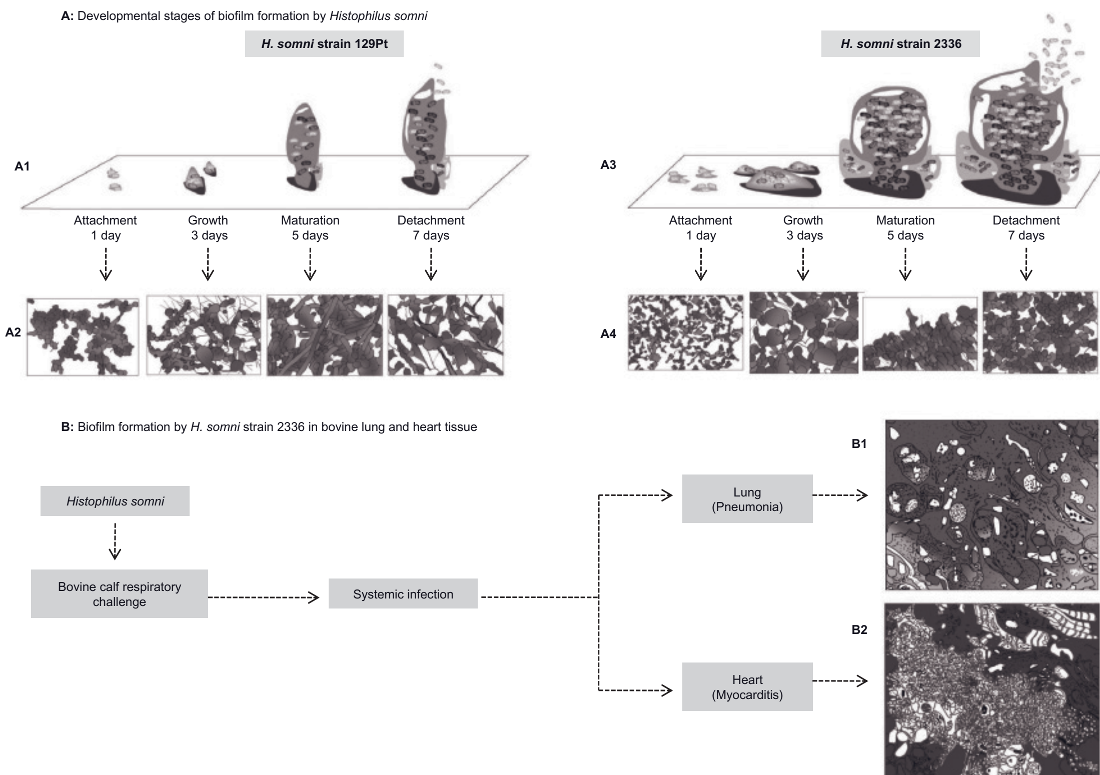

Figure 20.2. A: Comparison of developmental stages of biofilm formation by *H. somni* strains 129Pt (A1 & A2) and 2336 (A3 & A4); individual cells attach to the surface at 1 day; EPS is produced during growth at 3 days; biofilm structure develops and matures at 5 days; single cells are released from the biofilm at 7 days. B: Flow diagram of biofilm formation by *H. somni* strain 2336 in bovine lung (B1) and heart (B2).

the colonization of the respiratory tract or other aspects of virulence, and may provide a selective advantage for strains that cause systemic disease. In contrast, less virulent strain 129Pt only needs to colonize a specific site, which is a substantially different environment from that of diverse systemic tissue sites.

In Vivo. Bacterial biofilms form on mucosal surfaces, medical devices, and in organs where infections persist, such as the lungs (cystic fibrosis) and middle ear (otitis media; Donlan and Costerton 2002). Less is known about biofilm formation in animal infections, but such biofilms are believed to contribute to diseases such as mastitis due to *Streptococcus agalactiae* and *Staphylococcus aureus*, and pneumonia due to *M. haemolytica* and *Pasteurella multocida* (Clutterbuck et al. 2007). *H. somni* strain 2336 is also capable of forming a biofilm in cardiopulmonary tissue following experimental respiratory infection of the bovine host, as determined by various microscopic techniques and fluorescent *in situ* hybridization (Sandal et al. 2009). The biofilms are similar to those observed in H&E-stained sections from the infected middle ear of chinchillas infected with *H. influenzae* strain 2019 (Jurcisek et al. 2005), and bladders of C3H/HeJ mice infected with *E. coli* strain UTI89 (Anderson et al. 2003). Of interest is that *H. somni* biofilm formation in the myocardium is much more prevalent than in the lungs (fig. 20.2B; Sandal et al. 2009), which has been confirmed in an outbreak of *H. somni* myocarditis in feedlot cattle in Wyoming (O'Toole et al. 2009).

Exopolysaccharide and Biofilm Formation

Although many pathogenic members of the family *Pasteurellaceae* are encapsulated (Kilian 2005; Mutters et al. 2005; Olsen and Moller 2005), a capsule has not been identified on the surface of *H. somni*. Nonetheless, polysaccharide can be isolated from *H. somni* culture supernatant when the bacteria are grown under conditions of low oxygen tension (e.g., broth cultures without shaking) or other forms of stress. Compositional analysis revealed that the polysaccharide is composed predominantly of galactose and mannose, and is similar to yeast mannan. It is readily shed from the cell and is, therefore, an exopolysaccharide (EPS). Furthermore, it is most abundantly produced when the bacteria are grown as a biofilm. Antibodies to this antigen do not protect mice against systemic challenge with *H. somni.* Two loci in *H. somni* may be responsible for the synthesis of this EPS. EPS-A contains 17 putative genes whose predicted products have homology to proteins known to function in polysaccharide synthesis. This locus is similar to polysaccharide synthesis locus (*psl*), which is essential for biofilm formation in *Pseudomonas aeruginosa* strains PAO1 and ZK2870 (Jackson et al. 2004). The second locus (EPS-B) contains ORFs with similarity to genes that encode for phosphomannomutase (*manB*), flanked by UDP-glucose pyrophosphorylase (*galU*) and carbon storage regulator (*csrA*). This locus is similar to the *pel* gene cluster in *P. aeruginosa* responsible for synthesis of mannose- and galactose-rich EPS that contributes to the structure of biofilms in other *P. aeruginosa* strains (Ryder et al. 2007).

In vitro gene expression analysis of the EPS locus by quantitative reverse transcriptase polymerase chain reaction (PCR), following growth of strains 2336 and 129Pt in biofilm or planktonic phase, showed that 14 of the 17 genes of EPS-A are upregulated when *H. somni* is grown as a biofilm. In contrast, only five genes in this locus are up-regulated in strain 129Pt (Sandal and Inzana 2008). Thus, the *H. somni* EPS appears to be produced primarily under growth conditions that favor biofilm formation, and is a component of the biofilm matrix.

Protein Virulence Factors

Several surface proteins of *H. somni* are important in virulence. Convalescent phase serum recognizing a 40-kDa outer membrane protein (OMP) passively protects calves against experimental *H. somni*-induced pneumonia (Gogolewski et al. 1988). This OMP is surface-exposed and often induces the strongest antibody response of all the *H. somni* antigens. Theisen et al. (1992) cloned and characterized two 40-kDa proteins of *H. somni.* Both were lipid-modified, and they were designated LppA and LppB. A major OMP (MOMP) of 41 kDa, a heat-modifiable OMP (28 kDa at 60°C and 37 kDa at 100°C), and a 17.5-kDa OMP (reviewed in Corbeil et al. 2006) have also been implicated in virulence. The 41-kDa MOMP, or porin, undergoes antigenic variation, and even though the immune response to it is weak, it may be a factor in evasion of the host response (Tagawa et al. 2000). There is a minimal IgG-specific response to the MOMP in infected animals, but this antigen is immunodominant for the IgE response, which is

associated with more severe disease (Corbeil et al. 2006). Expression of a 31-kDa *H. somni* protein in *E. coli* yielded a product that lysed bovine erythrocytes, suggesting that it may also be a virulence factor (Won and Griffith 1993).

Another important virulence attribute is the ability to acquire iron in competition with the host. As noted, an iron-acquisition mechanism of *H. somni* involves binding of transferrin to a surface receptor. Two *H. somni* iron-repressible OMPs (transferrin-binding proteins, TbpA and TbpB) bind only bovine transferrin. This may be one reason for the narrow host specificity of *H. somni.* TbpA is predicted to be an integral (membrane spanning) OMP and may act as a gated pore for iron (Gray-Owen and Schryvers 1996). TbpB is a predicted peripheral (non-membrane-spanning) OMP that preferentially binds iron-loaded transferrin, suggesting a mechanism for optimization of iron acquisition (Retzer et al. 1998).

Hemolytic Activity

Some strains of *H. somni* produce a narrow zone of hemolysis on blood agar after >24 h incubation at 37°C. However, further incubation of some at 4°C yields more intense and distinct β-hemolysis. However, no *H. somni* hemolysin has been characterized. Hemolytic activity is not necessary for pathogenesis, since strain 2336 (used to reproduce pneumonia in calves) is nonhemolytic. It is not clear whether hemolytic activity plays a role in septicemia and its sequelae.

IgBPs

The IgBPs of *H. somni* are unique virulence factors related to other large surface proteins of gram-negative bacteria that cause respiratory or genital tract infections. The IgBPs bind bovine IgG2 by the Fc portion and comprise a peripheral membrane protein, p76, and a surface fibrillar network composed of a series of high-molecular-weight (HMW) proteins of varying sizes (Corbeil et al. 1997). Results of recent studies revealed that the HMW and p76 IgBPs are encoded by one ORF, *ibpA* (for IgBP A). Some strains from asymptomatic carriers lack the entire IbpA coding region (Cole et al. 1992) and are killed by bovine complement, whereas IbpA-positive virulent strains are resistant to complement-mediated killing. This resistance is critical to viability in the blood and, underlies septicemia and its sequelae. The N-terminal half of IbpA is homologous with *Bordetella pertussis* FhaB, including several adhesin domains (Tagawa et al. 2005). IbpA and the upstream IbpB proteins have homology to other large surface proteins belonging to the two-partner secretion family (Tagawa et al. 2005). The IgBPs may also play a role in endothelial cell adherence, since a mutant strain expressing truncated IbpA adhered less well than the wild type to bovine endothelial cells. This correlated with the lower virulence of the mutant for mice (Sanders et al. 2003). The C-terminal region of *H. somni* IbpA has homology to a new family of cysteine proteinases, including the conserved catalytic domain (Shao et al. 2002). Two direct repeats (DR2 and DR2) in the C-terminal region were recently shown to contain a toxin motif (Fic), which causes retraction and rounding of HeLa cells (Worby et al. 2009). This motif collapses the actin cytoskeleton by adenylating a tyrosine in the switch 1 region of Rho GTPases. Recently, Hoshinoo et al. (2009) showed that *H. somni* strain 2336 disrupted actin filament formation and inhibited phagocytosis by bovine macrophages, but an isogenic *ibpA* mutant did not. Thus, the IbpA fibrils on *H. somni* may be toxic for bovine macrophages.

Immunity

Vaccines have been available to protect against TME for many years and are also used to prevent *H.* somni-induced respiratory disease. However, the mechanisms of protection are not well understood. Several studies provide insight into the possible mechanisms of protective immunity. Passive immunization with convalescent serum protects against experimental bovine pneumonia (Gogolewski et al. 1987), suggesting that antibody is sufficient for immune defense and providing evidence that *H. somni* is not a typical facultative intracellular parasite. Protective convalescent phase serum recognizes predominantly the 78- and 40-kDa OMPs and the IgBPs described above. Furthermore, monospecific antibody to the 40-kDa OMP is also sufficient for protection (Gogolewski et al. 1988). This does not mean, however, that antibodies to other antigens would not also be protective. Immunoglobulin (Ig) class, subclass, or allotype may be as important as specificity in protection. These differences in Ig are due to differences in the primary amino acid sequence, usually of the heavy chain. Often these variations are in domains associated with secondary function

such as complement activation, opsonization, Fc binding to IgBPs, or resistance to proteinase cleavage. Each of these functions may be important in defense. IgG2 of appropriate specificity may be most critical for protection. Results of passive protection experiments with purified bovine IgG2 or IgG1 antibody versus the 40-kDa OMP tend to confirm the observation that IgG2 antibody to this OMP is protective (Corbeil et al. 1997). Since there are two allotypes of IgG2 ($IgG2^a$ and $IgG2^b$), which are inherited as codominant alleles, it was hypothesized that one allotype may function differently in protection than the other. This has been demonstrated for at least two functions related to defense against *H. somni*. First, $IgG2^b$, but not $IgG2^a$, binds to *H. somni* IgBPs by the Fc region (Bastida-Corcuera et al. 1999b). Second, $IgG2^b$ has twice the activity of $IgG2^a$ in complement activation (Bastida-Corcuera et al. 1999a). Complement is critical for defense against *H. somni*, so the inherited IgG2 allotype of an animal may contribute to susceptibility to *H. somni* infection. Interestingly, neonatal calves produce $IgG2^b$ several weeks later than $IgG2^a$ (Corbeil et al. 1997), possibly explaining the difference in susceptibility to *H. somni* pneumonia among young calves.

Other Ig classes are also stimulated by immunization or infection. High levels of IgA antibodies have been reported in bronchial lavage fluids of calves infected with *H. somni* (Gogolewski et al. 1989). Calves vaccinated with bacterins produced *H. somni*-specific IgE antibodies, and it was suggested that these antibodies may be related to more severe clinical disease (Ruby et al. 2000). Subsequent studies by Gershwin et al. (2005) showed that inoculation with BRSV followed by *H. somni* resulted in production of more IgE antibody to *H. somni* and more severe disease of longer duration. However, the specificity of this IgE response differs from the IgG responses of the same animals (Corbeil et al. 2006). Other factors are probably important in immune defenses against *H. somni*. The role of T cell-mediated immunity is yet to be defined. However, bovine IgG2 is associated with a Th1-type response in cattle, indicating that IgG2 antibody and T cell-mediated immunity are both involved in protection.

The ability of *H. somni* to evade these defense mechanisms also affects the outcome of the disease process. Both the LOS and the MOMP undergo antigenic variation as described, allowing the organism to escape immune defenses targeting the original epitopes. In addition, the organism binds host IgG2 to the surface IbpA and expresses oligosaccharides identical to glycosphingolipids on various cell types and decorates its surface with Neu5Ac. These processes may result in recognition of the organism as self, delaying the immune response. IbpA and Neu5Ac are also associated with resistance to complement-mediated killing, another strategy for evading defense. Suppression of phagocytic function is another way the pathogen avoids killing. However, even with these strategies of immune evasion, convalescent immunity or vaccination does result in some protection against *H. somni* disease. The balance between evasive mechanisms and host defenses determines whether or not infection progresses to disease.

Transformation and Mutagenesis

Mutagenesis of *H. somni* using allelic exchange is difficult because of the apparently tight restriction-modification (RM) system of this organism; only three genes have been mutated to date (Wu et al. 2000, Sanders et al. 2003, Hoshinoo et al. 2009). A vector useful for transforming *H. somni* was the inefficient broad host range shuttle plasmid pLS88. pNS3K, an improved version of pLS88, transforms *H. somni* strain 2336 100-fold more efficiently than its predecessor. The transformation efficiency is further increased when pNS3K is isolated from *H. somni* and retransformed into the same strain, supporting the role of the RM system in limiting transformation efficiency (Sandal et al. 2008).

Elucidation of the genome sequence of *H. somni* has enabled identification of the putative genes for RM systems, and in strain 2336 includes three genes (hsdR-hsdS-hsdM) of a Type I RM locus and two genes (*R.hsoI-M.hsoI*) of a Type II RM locus. The latter genes closely resemble the *Hin*P1I RM system of *H. influenzae* strain P1. The Type I RM locus in strain 129Pt contains only *hsdR*, a truncated version of *hsdS*, and no *hsdM*; a Type II RM locus is absent. The lack of a Type II RM locus and the presence of a type I RM locus may explain why this strain can be more easily transformed in the laboratory. The use of TypeOne™ Restriction Inhibitor (Epicentre) blocks Type I RM systems, thereby increasing transformation efficiency (Sandal et al. 2008).

Transposon mutants of *H. somni* strain 2336 can be generated using the EZ::Tn5™<KAN-2>Tnp Transposome™ system (Epicentre). Mutants can be

screened for a particular phenotype (e.g., alteration in biofilm formation) and compared with the parent (Sandal et al. 2009). Several mutants derived in this manner contain an insertion in a gene encoding a protein homologous to the filamentous hemagglutinin, Fha, a 220-kDa surface protein that acts as an adhesin to bind *Bordetella pertussis* to respiratory epithelial cells (Kajava et al. 2001; Inatsuka et al. 2005), human monocytes, and other cells (Relman et al. 1989). Fha comprises a membrane-anchored protein (FhaC) that is involved in the activation/secretion of the cognate hemagglutinin/adhesin (FhaB); as such, they are prototypes of a novel type of two-partner secretion system. The chromosome of *H. somni* strain 2336 contains four loci and 11 genes that encode proteins with homology to FhaB and three genes encoding proteins homologous to FhaC, including IbpA and IbpB. IbpA is expressed, but it is not clear whether the other 11 truncated FHA homologs are expressed. Since the first stage of biofilm formation is attachment, a mutation in *fhaB* would likely affect biofilm formation. Furthermore, FHA expression is up-regulated fourfold in cells forming a biofilm, as determined by real-time quantitative reverse transcriptase PCR (Sandal and Inzana 2008).

Sequence motifs required for efficient DNA uptake, called DNA uptake sequences (DUS, also known as uptake signal sequences [USS]), have been identified in *Neisseria sp.* (5′-GCCGTCTGAA-3′) and *H. influenzae* (5′-AAGTGCGGT-3′). The *H. influenzae* DNA USS is overrepresented in *H. ducreyi* and *H. somni*. The genome sequence of *H. somni* strain 129Pt has ~1244 potential USS. Sixty-two percent (776) of these sites are within coding sequences and the rest are in intergenic regions (Bakkali et al. 2004). Strains 129Pt and 2336 have genes involved in DNA uptake and transformation (Redfield et al. 2006), and can be successfully transformed using homologous DNA containing a kanamycin resistance gene as a marker, following a modification of the standard transformation protocol used for *H. influenzae* (Barack et al. 1991). Thus, natural transformation may prove to be an efficient method of mutagenesis for *H. somni.*

Summary

It is now clear that virulent strains of *H. somni* produce a multitude of virulence factors primarily aimed at colonization and evasion of host defenses, and which may result in toxicity. Some serum-sensitive strains from the male genital tract lack many of these factors, and therefore may be restricted to colonization of a particular niche within the host. Virulent strains apparently cause disease primarily through colonization of multiple tissue sites, the capacity to cause apoptosis or cell retraction, penetration of epithelial and endothelial cells, dissemination, and resistance to host defenses. Disease likely occurs as a consequence of cytotoxicity and host inflammation, due in part to the presence of endotoxin and IbpA, which compromise epithelial, endothelial, and inflammatory cell defenses. The subsequent infiltration of PMN and macrophages to sites of bacterial infection results in additional lesion formation.

HAEMOPHILUS PARASUIS

Overview

Haemophilus parasuis is a small, nonmotile, pleomorphic gram-negative that often colonizes the upper respiratory tract of swine. It is the etiologic agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis, and meningitis (Amano et al. 1994), or acute pneumonia and septicemia without polyserositis (Peet et al. 1983). Fibrinous polyserositis and arthritis caused by *H. parasuis* is usually diagnosed on the basis of herd history, clinical signs, necropsy findings, and bacterial isolation/detection (Oliveira and Pijoan 2004). The course of disease is particularly serious in specific-pathogen-free (SPF) herds and conventional herds with good health status. Clinical disease particularly affects young animals exposed to previous viral infection or stress. The same can occur in an infected herd when a new, antigenically distinct strain is introduced (Oliveira and Pijoan 2004). *H. parasuis* endotoxin (LOS) causes disseminated intravascular coagulation (Peet et al. 1983; Amano et al. 1994).

Recently developed genotyping techniques have been used to characterize the virulence of *H. parasuis* (De la Puente Redondo et al. 2003; Oliveira et al. 2003, 2006; Del Rio et al. 2006). Fifteen capsular serovars have been recognized, and the virulence of serovar reference strains has been determined in SPF piglets (Kielstein and Rapp-Gabrielson 1992). Serovar reference strains 1, 5, 10, 12, 13, and 14 are considered highly virulent, causing greater morbidity or mortality in pigs within 4 days post challenge. Serovars 2, 4, and 15 cause polyserositis

without mortality and may be of intermediate virulence. Serovars 3, 6, 7, 8, 9, and 11 did not cause clinical signs in pigs and are considered relatively avirulent (Kielstein and Rapp-Gabrielson 1992; Amano et al. 1994). There is also variation in virulence within serovars (Oliveira and Pijoan 2004).

Virulence Factors

Identification of potential *H. parasuis* virulence factors and mechanisms of pathogenesis has been an active area of investigation (Hill et al. 2003; Lichtensteiger and Vimr 2003; Tadjine et al. 2004; Melnikow et al. 2005; Vanier et al. 2006). Virulence factors include neuraminidase (Lichtensteiger and Vimr 1997), transferrin-binding proteins (Charland et al. 1995), capsule, LOS (Biberstein 1990), and fimbriae (Munch et al. 1992). No gene encoding neuraminidase has been identified, but genes encoding fimbriae and transferrin-binding proteins are apparently present in all strains (Metcalf and MacInnes 2007). Vanier et al. (2006) demonstrated that virulent strains of *H. parasuis* invade endothelial cells, but the factors involved have yet to be determined. Microarrays and reverse transcriptase PCR have also been used to examine gene expression under growth conditions that mimic *in vivo* conditions (Hill et al. 2003; Melnikow et al. 2005; Jin et al. 2008). Like *H. influenzae* and *H. somni*, *H. parasuis* forms biofilms; however, avirulent serovars appear to form more substantial biofilms than do virulent serovars (Jin et al. 2006). Thus, the relationship between biofilm formation and *H. parasuis* pathogenesis is questionable.

Capsule

There are at least 15 serovars of *H. parasuis* based on immunodiffusion testing with heat-stable antigens (Kielstein and Rapp-Gabrielson 1992; Rapp-Gabrielson and Gabrielson 1992). Nearly 80% of North American isolates fall into seven serovars and more than 15% are untypable. The identity of the serovar-specific antigen has not been confirmed, but it is probably the capsular polysaccharide (CP). The presence of CP on *H. parasuis* has been confirmed (Morozumi and Nicolet 1986), but a CP has not been purified or chemically characterized. Like typeable *H. influenzae*, and unlike *H. somni*, *H. parasuis* appears to form a typical CP. The role of CP in virulence has not been specifically investigated, but it probably acts to protect the bacterium from host defenses. CPs are most often high-molecular-weight, negatively charged carbohydrate polymers, usually consisting of repeating units of two or three monosaccharide residues. Phosphate, carboxyl, or other acidic groups give the CPs a negative charge. The CP is shed by the bacterium into its surrounding matrix, but it is also anchored to the cell membrane by a lipid (Gotschlich et al. 1981). The CP is a T cell-independent antigen, and as a result may be poorly immunogenic, especially in young animals. CPs normally do not activate complement; in the absence of specific antibodies, the bacterium can evade complement-mediated killing and phagocytosis. Each serotype produces a chemically and antigenically distinct CP, and antibody to one CP serotype usually will not protect against another serovar (Robbins et al. 1980).

LOS

H. parasuis produces LOS that does not undergo phase variable expression (Inzana and Corbeil 2004). Several reports have proposed that LPS or LOS of gram-negative meningeal pathogens, such as *E. coli*, *Neisseria meningitidis*, and *Haemophilus* spp. (Humphries et al. 2005; Kuckleburg et al. 2005; Metkar et al. 2007), are major virulence factors. LOS can induce apoptosis of endothelial and epithelial cells (Bannerman et al. 2002; Sylte et al. 2001; Chin et al. 2006), cause release of pro-inflammatory mediators (Khair et al. 1996), and is involved in bacterial adhesion to host cells (Alfa and DeGagne 1997; Swords et al. 2000). *H. influenzae* LOS causes marked cytotoxicity to bovine brain microvascular endothelial cells (BMEC) (Tunkel and Scheld 1993). Moreover, *H. somni* LOS induces apoptosis of bovine endothelial cells (Sylte et al. 2001). However, little is known about the biological activity of LOS from *H. parasuis*. Zucker et al. (1994) did not detect significant differences in the amount of LOS produced by virulent and avirulent *H. parasuis* strains, but LOS from *H. parasuis* does exert endotoxin-like activity (Raetz and Whitfield 2002). Amano et al. (1997) proposed that *H. parasuis* endotoxins are linked to endotoxic shock, enhancement of clinical signs, and death of pigs with septicemia, suggesting that the LOS might play an important role in pathogenesis.

In gram-negative infections, cytokine-related disruption of the BBB is mediated through LPS/LOS activity (Verma et al. 2006). *H. parasuis* LOS induces TNF-α production by a mouse macrophage

cell line (Ogikubo et al. 1999). *H. parasuis* induces brain endothelial cells to release pro-inflammatory cytokines, which in turn may play an important role in local inflammation as well as in increasing BBB permeability. However, as with *N. meningitidis*, LOS is only partially responsible for *H. parasuis*-induced cytokine production; other cell-associated or heat-resistant bacterial component(s) are also involved (Bouchet et al. 2008). Further studies are needed to identify other surface structures involved in porcine brain microvascular endothelial cell (PBMEC) stimulation by *H. parasuis*.

Haemophilus parasuis LOS is involved in adhesion to newborn pig tracheal (NPTr) cells (Bouchet et al. 2008). Furthermore, *H. parasuis* whole cells can induce NPTr cell apoptosis in a caspase-3-dependent fashion, but the LOS is likely not involved in this process. *H. parasuis* whole cells, and to a lesser extent its LOS, stimulate IL-8 and IL-6 release by NPTr cells. Serotype 4 field isolates induce higher levels of these mediators than serotype 5 isolates, suggesting that bacterial adhesion, induction of apoptosis and cytokine release are important events for *H. parasuis* colonization. However, LOS appears to have a limited role in these processes. Therefore, the contribution of *H. parasuis* LOS to virulence and the disease process remains to be defined.

Protein Antigens

There is little information on the role of proteins in the virulence of *H. parasuis*. Maclnnes and Desrosiers (1999) reported no known toxins. Schaller et al. (2000) showed that *H. parasuis* is devoid of Apx I, Apx II, and Apx III toxin genes, but fimbriae and a neuraminidase are present (MacInnes and Desrosiers 1999). The relationship of these antigens to virulence is not clear, but certain OMP profiles seem to be related to virulence (Ruiz et al. 2001), and one OMP has been studied in more detail. The *tbpA* gene encoding for Tbp1 (transferrin-binding protein) is species specific. Restriction fragment length polymorphism analysis revealed considerable heterogeneity within this ORF, making it useful for typing and for epidemiologic tracing (De la Puente Redondo et al. 2003). In *A. pleuropneumoniae*, Tbp1 is an integral OMP that is involved in the uptake of iron. If the protein has a similar location and function in the closely related *H. parasuis*, Tbp1 may be involved in the acquisition of iron from transferrin, and therefore be required for growth in the host.

Host defense mechanisms against *H. parasuis* infection include killing by alveolar macrophages, which can be compromised by prior infection with a virus such as porcine reproductive and respiratory syndrome virus (PRRSV; Solana et al. 1998). However, systemic vaccination with killed *H. parasuis* and aluminum hydroxide adjuvant is protective. Cross-protection has been demonstrated with some strains, but not with others (Bak and Riising 2002).

Other Virulence Factors

Metcalf and MacInnes used a differential display technique to identify *H. parasuis* genes expressed upon iron restriction and during growth in cerebrospinal fluid. Several genes were identified, but they are present in all serovars. Therefore, if these genes are responsible for any differences in virulence, it would likely be due to differences in expression. One such example is a haloacid hydrogenase, which is up-regulated in virulent reference strains (Metcalf and MacInnes 2007). Another potential factor is a putative hemolysin that is encoded on an operon (hhdBA) present in half of the serovar reference strains described as virulent, but not in those reference strains reported to cause no disease in animal infection experiments (Sack and Baltes 2009). Electroporation with a native plasmid (Lancashire et al. 2005) and natural transformation (Bigas et al. 2005) have been used for genetic manipulation of *H. parasuis*. Use of either genetic system for recombinant expression of specific genes and for production of defined mutants may establish the role of these genes in virulence.

Recently, the genome of *H. parasuis* serovar 5 strain SH0165 (isolated from a pig with Glässer's disease) has been sequenced (Yue et al. 2009). Numerous virulence-associated putative genes are included in the SH0165 genome, such as the typical two-cluster *cdtABC* toxin loci; they encode cytolethal distending toxin, which initiates a eukaryotic cell cycle block at the G_2 stage prior to mitosis. An ORF with homology to *oapA* encodes a surface-associated lipoprotein, and may play an important role in adherence. This protein is responsible for the transparent colony phenotype of *H. influenzae* and is required for efficient colonization of the nasopharynx (Weiser et al. 1995; Prasadarao et al. 1999). The SH0165 genome includes an intact type IV secretory system locus named *pilABCD*, and several putative autotransporter genes, such as *espP*,

aidA, and *sphB*. However, many other homologs of virulence-related genes are defined as pseudogenes. For example, there are 11 *sclB* genes encoding a triple-helix-repeat containing collagen, which in *H. ducreyi* encode for a large adhesin (Challacombe et al. 2007), but are inactive in *H. parasuis* as a result of frameshifts. *sclB* genes encoding a triple-helix-repeat-containing collagen (a large adhesin identified in *H. ducreyi*) are inactive as a result of frameshifts (Challacombe et al. 2007). Possible changes in the *H. parasuis* lifestyle led to modifications in the utility of some genes. The high proportion of pseudogenes is related both to the dispensability of previously useful genes in the porcine upper respiratory tract and to a population structure that promotes the maintenance of deleterious mutations (Ochman and Davalos 2006).

AVIBACTERIUM PARAGALLINARUM

Overview

Phylogenetic analysis demonstrated that *Haemophilus paragallinarum*, *Pasteurella gallinarum*, *Prunus avium*, and *Pasteurella volantium* formed a monophyletic group with a minimum of 96.8% sequence similarity. Therefore, each of these strains has been proposed to be transferred to the new genus *Avibacterium* as *Avibacterium paragallinarum* comb. nov., *Av. gallinarum* gen. nov., comb. nov., *Av. avium* comb. nov., and *Av. volantium* comb. nov. (Blackall et al. 2005). *Avibacterium* (*H.*) *paragallinarum* is the causative agent of avian infectious coryza, an upper respiratory disease of poultry. Clinical signs include facial edema, lacrimation, anorexia, and rhinitis resulting from inflammation of the turbinates and sinus epithelium, and acute air sacculitis. *Av. paragallinarum* can cause primary disease, but the infection is usually more severe following viral or mycoplasma infection. The disease occurs worldwide, and causes economic losses through increased culls and a marked drop in egg production (from 10% to more than 40%; Blackall 1999). *Av. paragallinarum* was typed according to the Page scheme into serovars A, B, and C by hemagglutination inhibition (HI) tests (Blackall et al. 1990). All three Page serovars have been isolated from infected chickens around the world (Blackall 1999), and Page serovars A and C have been isolated from a number of outbreaks of infectious coryza in several parts of Taiwan (Terry et al. 2003). These serotypes have been the basis for development of both blocking ELISA and hemagglutination inhibition assays for detection of antibodies to *Av. paragallinarum* (Sun et al. 2007).

Virulence Factors

Several factors have been associated with the virulence of *Av. paragallinarum*, including capsule, LPS, and hemmagglutinin (HA).

Capsule

CP has clearly been associated with virulence (as well as colony iridescence and smoothness), and the ability of strains to cause experimental infectious coryza is related to the amount of CP produced (Sawata and Kume 1983). Early reports suggested that the CP may also be involved in adherence of the bacteria to the host respiratory tract, since encapsulated bacteria, but not nonencapsulated bacteria, were associated with upper respiratory tract lesions (Sawata et al. 1985).

HA

HA is considered the most important of the known virulence attributes due to its participation in tissue adhesion (Hobb et al. 2002) and because mutants lacking HA are avirulent (Yamaguchi et al. 1993). HA is a good immunogen, and high titers of anti-HA antibodies have been considered indicators of protection against infectious coryza (Takagi et al. 1993). Purified HA protects against infection with the homologous strain. A modified Kume serotyping scheme relies on HI to define serovars A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4 (Soriano et al. 2001).

The purified HA of *Av. paragallinarum* is a 39-kDa OMP. Hobb et al. (2002) cloned and sequenced the hemagglutinin gene, *hag*, which is similar to the P5 family of OMPs of other *Pasteurellaceae.* Variation in the amino acid sequence among strains of different serovars is small. Recently, Barnard et al. (2008) reported the partial purification and characterization of the HA-L hemagglutinin from *Av. paragallinarum* strain 46-C3, a heat-sensitive, trypsin-sensitive HA that is the serovar-specific HA in this organism. This protein shares similarities to other types of adhesins found in gram-negative bacteria.

Serovar-specific immunity is induced by bacterins. Different serovars are associated with different geographic locations. Vaccine failures in a given geographic region may result from emergence of antigenic variants (serovars not previously present in that area) or change in prevalence of various

serovars. Therefore, it is critical to include the local serovars in vaccines used in any given location.

Iron-Acquisition Proteins

The response to iron deficiency in *Av. paragallinarum* has not been well studied. OMPs of 53, 62, 66, and 94 kDa were identified in iron-repressed *Av. paragallinarum* (Ogunnariwo and Schryvers 1992). The 53- and 94-kDa proteins were further purified and identified as specific receptors for chicken or turkey ovotransferrin (Ogunnariwo and Schryvers 1996), suggesting this protein's participation in iron acquisition. Recently, Abascal et al. (2009) reported characterization of over-expressed OMPs in *Av. paragallinarum* cultured in iron-restricted medium and the presence of a *fur* gene within its genome. Mass spectroscopy analysis of the iron-restricted OMPs indicated that the 94-kDa protein has homology to a transferrin receptor (TbpA) from *A. pleuropneumoniae* and to TbpA from *H. parasuis*. The 66-kDa protein has homology to outer membrane receptor proteins, predominantly those involved in iron transport in *A. pleuropneumoniae* serotype 1 strain 4074. The 62-kDa protein is similar to the transferrin-binding protein TbpB of *A. pleuropneumoniae* serotype 7.

A candidate *fur* gene of *Av. paragallinarum* has 94% identity with Fur sequences of *A. pleuropneumoniae* and *H. ducreyi*. The *Av. paragallinarum*-deduced Fur protein (17.8 kDa) amino acid sequence contains the N-terminal helix-turn-helix DNA-binding domain and the two iron-binding sites in its C-terminal end, which is typical of other Fur proteins (Pohl et al. 2003). Further study of the iron restriction-induced proteins and the mechanism regulating their expression may lead to an understanding of the responses of *Av. paragallinarum* necessary for survival in the iron-restricted environment of host mucosal surfaces.

Antibiotic Resistance and Putative Virulence Plasmids

Hsu et al. (2007) serotyped 18 *Av. paragallinarum* isolates collected in Taiwan from 1990 to 2003 and tested them for resistance to antimicrobial agents. More than 75% of the isolates are resistant to neomycin, streptomycin, and erythromycin. The most common resistance (15 isolates, 83.3%) is to neomycin–streptomycin. About 72% of isolates contained the plasmids pYMH5 and/or pA14. Plasmid pYMH5 encodes streptomycin, sulfonamide, kanamycin, and neomycin resistance genes, and has significant homology to the broad host range plasmid pLS88, which is a native plasmid of *H. ducreyi*. Plasmid pYMH5 is the first multidrug-resistant plasmid reported in *Av. paragallinarum*, and it may facilitate the spread of antibiotic-resistance genes among bacteria. Plasmid pA14 encodes a putative MglA protein and RNase II, both of which might be associated with virulence. MglA is an ABC-type sugar transport protein that is responsible for carbohydrate transport and metabolism. The ABC domain 2 of MglA can only transport monosaccharides (MOS). MglA may also be involved in the pathogenesis of *Brucella abortus* infections (Jacob et al. 2006), the motility of *Myxococcus xanthus* (Hartzell and Kaiser 1991), and the transcription of virulence factors of *Francisella tularensis* (Lauriano et al. 2004). RNase II is a member of the RNR superfamily of exoribonucleases (Cairrao et al. 2003). The RNase II-related protein VacB is associated with virulence in *Shigella flexneri* and *E. coli* (Cheng et al. 1998), and colonization in *Burkholderia* spp. (Ruiz-Lozano and Bonfante 2000). Another plasmid described in *Av. paragallinarum* strains is p250, from an isolate (HP250) obtained in Taiwan (Blackall et al. 1991). It has been fully sequenced and carries a hemocin-producing locus that expresses a protein capable of killing a wide range of other gram-negative bacteria (Terry et al. 2003). In 17 of 19 plasmid-free strains, this hemocin locus is in the bacterial chromosome (Terry et al. 2003). Thus, the putative virulence plasmid pA14 and hemocin-like activity in *Av. paragallinarum* may be involved in virulence.

NAD Dependence

Another factor that may be linked to virulence is dependence upon NAD for growth. Most strains are NAD-dependent, but NAD-independent strains have been detected. Serovar C-3 NAD-dependent isolates were much more virulent than C-3 NAD-independent isolates (Taole et al. 2002). To test the hypothesis that NAD independence was plasmid-borne, these authors transformed an NAD-dependent strain of *Av. paragallinarum* with plasmid DNA from an NAD-independent C-3 isolate. The resulting transformant was NAD-independent and was reduced in virulence for chickens. The mechanism for reduced virulence is not clear. Many species of the *Pasteurellaceae* require NAD for growth, but NAD dependence has not been linked to virulence in other species.

There is little information regarding the endotoxin of *Av. paragallinarum*, but this component probably is the reported heat-stable toxin (Iritani

et al. 1981). The OMPs of *Av. paragallinarum* were similar among 15 isolates, although two SDS-PAGE profiles could be identified. One protein is heat modifiable, and many of the proteins, including conserved proteins, are recognized by immune serum to one isolate (Blackall et al. 1990). The role of these proteins in virulence, however, has not been determined.

CONCLUSIONS

The basis for the virulence of *H. parasuis* and *Av. paragallinarum* is not established, and it is not clear how these opportunistic pathogens cause disease in their specific hosts. Both species are host-specific and encapsulated, produce endotoxin but no known exotoxins, and can cause serious disease. Therefore, it appears that these species, like *H. somni*, can evade host defenses and cause disease associated with inflammation. Additional research, perhaps based on further analysis of genome sequences of virulent isolates, is required to determine what factors contribute to resistance to host defenses, to colonization, and to host cell damage.

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21
Bordetella

K. Register and E. Harvill

INTRODUCTION

The genus *Bordetella* includes eight formally recognized species, of which *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Bordetella avium*, and *Bordetella hinzii* are of veterinary interest. *Bordetella pertussis*, the type species, is an obligate human pathogen and the causative agent of whooping cough. *B. parapertussis*, *B. bronchiseptica*, and *B. avium* are also associated with respiratory disease. *B. parapertussis* comprises two genetically and phenotypically distinct subgroups, one causing a pertussis-like illness in humans and the other a chronic, nonprogressive pneumonia in sheep. *B. bronchiseptica* causes tracheobronchitis (kennel cough) in dogs, bronchopneumonia in numerous laboratory, companion, and wild animals, and neonatal pneumonia in piglets and is an important contributor to swine atrophic rhinitis. Infection is often chronic and/or asymptomatic. The organism also rarely causes disease in humans. *B. avium* is the etiologic agent of turkey coryza and, with the exception of two recent isolations from humans (Spilker et al. 2008; Harrington et al. 2009), has been found exclusively in avian hosts. *B. hinzii*, recognized as a species since 1994, has been identified primarily from humans and poultry. It is an opportunistic pathogen in immunocompromised humans, and some strains induce mild to moderate respiratory disease in turkeys (Register and Kunkle 2009).

The remaining species of the genus, recognized since 1995 or later, include *Bordetella holmesii*, *Bordetella petrii*, and *Bordetella trematum*. All are associated with opportunistic human infections of the respiratory tract, blood, or other body sites. An additional species isolated from humans, *Bordetella ansorpii*, has been described but not yet accorded standing in nomenclature (www.bacterio.cict.fr/nonvalid.html).

CHARACTERISTICS OF THE GENUS

Bordetella are gram-negative coccobacilli of the family *Alcaligenaceae* and closely related to *Achromobacter* and *Alcaligenes* (Gerlach et al. 2001). Comparative studies suggest that *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*, the so-called "*B. bronchiseptica* cluster," or "classical" *Bordetella*, are highly clonal and represent subspecies of a single species. *B. bronchiseptica* has been proposed to be the ancestral progenitor from which two independent, host-restricted clones, *B. pertussis* and *B. parapertussis*, evolved. Genome sequences recently obtained from these species support this hypothesis (Parkhill et al. 2003).

All *Bordetella* grow under aerobic conditions; only *B. petrii* also grows anaerobically. The *Bordetella* are unable to utilize carbohydrates and depend on amino and carboxylic acids as primary carbon and energy sources. Growth of *B. pertussis* is inhibited by fatty acids and other components found in many media, and specialized formulations are required for isolation. Most other *Bordetella* species grow readily on a variety of agar media, including blood or MacConkey agar, and form mature colonies in 1–3 days. *Bordetella* of veterinary importance are resistant to a number of antibiotics, and selective media are preferred for isolation since overgrowth of other, faster growing bacteria

present in higher numbers otherwise interfere with detection. The optimal temperature for cultivation of *Bordetella* is 37°C, although all but *B. pertussis* grow reasonably well at temperatures as low as 25°C. Speciation of *Bordetella* isolates may generally be accomplished by consideration of the host of origin and the results of traditional biochemical tests (Songer and Post 2005). Polymerase chain reaction (PCR) assays for identification of *B. bronchiseptica* and *B. avium* have been favorably evaluated for use by veterinary diagnostic laboratories (Register and Yersin 2005; Register and DeJong 2006), but clinical validation has so far not been reported.

SOURCES OF THE BACTERIA

Bordetella species of veterinary interest are associated with mammals and/or birds and have a worldwide distribution. In their natural hosts, they inhabit the respiratory tract and are spread by direct contact or aerosol. *B. bronchiseptica* is most commonly found in pigs, dogs, rabbits, guinea pigs, and cats and has also been isolated from poultry and many other farm, companion, or wild animals. There is evidence for occasional transmission across host species, particularly between dogs and cats (Gaskell et al. 2006), but the full extent to which this occurs is unclear. Potential sources of *B. avium* include many species of domesticated and wild birds (Hopkins et al. 1990; Raffel et al. 2002). Transmission from free-living avian populations to poultry production farms has yet to be demonstrated. Poultry are the primary natural hosts of *B. hinzii*; isolation from a rabbit and a mouse has also been reported (Register et al. 2003; Hayashimoto et al. 2008). *B. parapertussis* is found exclusively in sheep and humans, and the two host-specific subgroups are not known to cross species.

Many animal hosts may be asymptomatically infected with *Bordetella*, and these carriers are a significant source of exposure and spread, especially under conditions associated with intensive food animal production or companion animal shelters. Excepting the human-adapted lineage of *B. parapertussis*, *Bordetella* species of veterinary significance only rarely cause disease in healthy adult humans. It is unclear whether humans may act as asymptomatic carriers of *B. bronchiseptica* following exposure to infected or immunized animals. There are only a few reports of adverse consequences secondary to such exposure, but this is an area in need of further investigation (Berkelman 2003).

VIRULENCE FACTORS

The classical *Bordetella* share an array of known or putative virulence genes, some of which are species specific. Most can be broadly categorized as colonization factors or toxins; additional products that otherwise alter host functions or assist in survival have also been described. Historically, most investigations have focused on *B. pertussis* due to its importance as a human pathogen. The extensive sequence identity generally shared among virulence gene orthologs of the classical *Bordetella* suggests that the corresponding proteins may perform equivalent functions, and related investigations generally confirm this. Nonetheless, the importance and function of some virulence factors differ among species, likely reflecting host-specific adaptation, and cross-species inferences should be viewed as presumptive. Initial efforts to identify common virulence factors in *B. avium* were largely unsuccessful. More recent studies reveal that these failures are attributable to both limited DNA sequence similarity of orthologous genes and diversity in the complement of *B. avium* virulence factors (Spears et al. 2003; Sebaihia et al. 2006). Information is limited regarding virulence factor identity and function in *B. hinzii*, but a few classical *Bordetella* virulence factors have been detected in some, but not all, isolates (Gerlach et al. 2001; Donato et al. 2005). Table 21.1 summarizes existing knowledge with respect to major virulence factors expressed by *Bordetella* species of veterinary interest; *B. pertussis* is included for comparison.

Filamentous Hemagglutinin

Filamentous hemagglutinin (FHA), encoded by the *fhaB* gene, is a large, rod-shaped, ~220 kDa protein that functions as a major adhesin and is both associated with the bacterial cell surface and released into the culture medium during growth *in vitro*. Several distinct domains of FHA have been implicated in attachment to a variety of cell and tissue types (Mattoo and Cherry 2005). These include a carbohydrate recognition domain involved in binding to ciliated cells and macrophages, a heparin-binding domain of potential importance in the initial interaction with components of the extracellular matrix of the respiratory tract (also involved in hemagglutination), and an Arg-Gly-Asp (RGD) amino acid triplet that promotes binding to leukocytes, stimulates phagocytosis, and alters leukocyte signaling such that bacterial binding via complement receptor type

Table 21.1. Virulence Factor Expression in *B. pertussis* as Compared with Species of Veterinary Importance

Virulence factor	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*	*B. hinzii*
BvgAS	+	+	+	+	+
Filamentous hemagglutinin	+	+	+	+	–
Fimbriae	+	+	+	+	ND
Pertactin	+	+	+	–	–
BrkA	+	+	+	–	ND
BatB	–	+	+	–	ND
Adenylate cyclase toxin	+	+	+	–	+/–[a]
Dermonecrotic toxin	+	+	+	+	–
Tracheal cytotoxin	+	+	+	+	ND
Osteotoxin	+	ND	+	+	ND
Alcaligen	+	+	+	–	ND
Enterobactin receptor	+	+	+	+	ND
Heme uptake system	+	+	+	+	ND
Type III secretion system	+[b]	+	+	–	–
Pertussis toxin	+	–	–	–	–

[a]Results differ for the only two strains so far evaluated.
[b]Demonstrated only in low-passage isolates.
ND = not determined.

3 (CR3) is increased. FHA is essential for tracheal colonization by *B. bronchiseptica* in rats, although one or more other adhesins are also required (Cotter et al. 1998), and it plays a primary, but not exclusive, role in biofilm formation (discussed later). The functional domains of the predicted proteins are highly conserved across the classical *Bordetella* species, but alterations are also present that may underlie, in part, differences in host specificity, cell- and tissue-specific colonization, and host receptor binding.

The *fhaB* gene directs synthesis of a 367-kDa precursor that is modified at both the N- and C-termini to produce a 220-kDa mature protein. An accessory gene in the same operon, *fhaC*, is required for translocation of FHA across the outer membrane by the two-partner secretion (TPS) pathway. Recent studies provide a detailed model for TPS transport and demonstrate that the C-terminus of cell-associated FHA is critically important for function *in vivo*, likely due to its orientation away from the cell surface and consequent availability for binding host cell receptors (Hodak et al. 2006; Mazar and Cotter 2006).

The *B. avium fhaB* and *fhaC* orthologs are required for virulence in turkeys, but the precise role of FHA in infection is unclear since mutation of either gene does not significantly impair attachment to tracheal rings (Spears et al. 2003; Sebaihia et al. 2006). Hemagglutination by *B. avium*, also associated with virulence, is likewise not a function of FHA but has been attributed to the proteins encoded by the *hagA*/*hagB* genes, which are novel among *Bordetella* but share some similarity with *fhaB*/*fhaC* (Sebaihia et al. 2006).

Additional genes encoding proteins highly similar to FHA have been identified among *Bordetella*, including those of veterinary importance (Julio and Cotter 2005; Sebaihia et al. 2006). None have so far been implicated in attachment or virulence.

Fimbriae

Fimbriae, produced by a variety of bacteria including *Bordetella*, are hair-like filaments extending from the cell surface that mediate attachment. The fimbrial shaft is composed of a repeating major subunit protein. Six major fimbrial subunit genes, scattered throughout the chromosome, have been identified among the classical *Bordetella*, but expression of only two, *fim2* and *fim3*, is shared among all species in the group (Mattoo and Cherry 2005). Fimbriae are highly immunogenic, and the Fim2 and Fim3 proteins are the basis for the serological typing system for *B. pertussis*.

The remaining major subunit genes are either absent from one or more species or present as pseudogenes that are not expressed due to a frameshift in the coding region or a deletion in the promoter. For example, the *fimA* subunit gene is found in all three species, but expression appears to be limited to *B. bronchiseptica* and ovine strains of *B. parapertussis* (Parkhill et al. 2003; Mattoo and Cherry 2005). The *fimA* gene is found within the *fimABCD* cluster, itself located between the *fhaB* and *fhaC* genes. *FimB* and *fimC* are involved in transport and assembly of the fimbrial structural proteins; *fimD* encodes the minor structural protein that forms the fimbrial tip. Synthesis of FHA and fimbriae is tightly coupled since the *fimBCDfhaC* genes are co-transcribed from a single promoter upstream of *fimB*.

In addition to control by the *bvgAS* locus (see discussion later), expression of most fimbriae is regulated by phase variation, a random, reversible event resulting from slipped-strand mispairing during DNA replication. Misalignment of the parent and daughter strands along a continuous stretch of cytosine residues (C stretch) upstream of the promoter leads to alterations in the length of the C stretch, thereby abolishing or activating transcription. Since the fimbrial structural genes are unlinked on the chromosome, phase variation of those with a C stretch occurs independently, and any combination of phase-variable fimbriae may be expressed simultaneously. On-off switching of fimbrial expression may be important for the maintenance of fimbrial function in the face of an immune response or other host or environmental selective pressures.

Bordetella avium also produces fimbriae, and 11 putative fimbrial subunit genes have been identified (Spears et al. 2003; Sebaihia et al. 2006). All but one, an ortholog of *fimA*, are novel to this species. The *B. avium* fimbrial structural subunit genes have no C stretch, but several contain a repeated sequence at the 5' end, which is potentially amenable to phase variation.

Both the major and minor fimbrial subunit proteins bind to sulfated sugars common on cell surfaces and in the mucus and extracellular matrix of the respiratory tract. Additionally, FimD binds to the monocyte receptor VLA-5, leading to increased FHA-mediated attachment through interaction with CR3. Although not requisite for colonization of the nasal cavity, fimbriae play an important role in colonization of the trachea and are required for persistence at that site.

Pertactin and Other Autotransporter Proteins

Pertactin is an outer membrane protein adhesin initially described as a protective immunogen in mice challenged with *B. bronchiseptica* and later shown to have a similar effect in pigs (Kobisch and Novotny 1990). The pertactin gene *prn* is found in all species of the *B. bronchiseptica* cluster but not in *B. avium* (Sebaihia et al. 2006). Pertactin is a member of the autotransporter family of proteins, known to play important roles in the pathogenesis of many gram-negative bacteria (Wells et al. 2007). Autotransporter proteins are characterized by a conserved C-terminal translocator domain that forms a pore through which the N-terminal passenger domain is directed for transit across the outer membrane. Following cleavage, the passenger domain remains cell-associated through a noncovalent interaction with the translocator, which is itself retained within the membrane. Mature pertactin, the fully processed N-terminal domain, migrates at ~68–70 kDa depending on the species of *Bordetella* that produces it.

The protein contains two regions of repetitive amino acids that form separate loops extending from a helical backbone (Emsley et al. 1996). Alterations in the size of pertactin are due to variance in the number of repeat units at these sites (Boursaux-Eude and Guiso 2000; Register 2001). Immunoprotective epitopes reside within both regions, and it has been postulated that repeat sequence polymorphism serves as a mechanism for immune evasion, with the antigenically variable immunodominant epitopes acting as decoys to prevent recognition of functional domains (Hijnen et al. 2007). Located adjacent to the N-proximal repeat is an RGD motif involved in binding to Chinese hamster ovary (CHO) cells. Numerous other *in vitro* studies suggest a role for pertactin in attachment, but direct evidence for its specific role *in vivo* is lacking, and it may be that pertactin functions as a subordinate or accessory adhesin during infection (Hibrand-Saint Oyant et al. 2005).

Twenty-one additional predicted or known autotransporter genes have been identified among the classical *Bordetella* (Parkhill et al. 2003; Wells et al. 2007), with some absent from or not expressed by all species. The BrkA autotransporter confers resistance to complement-mediated killing in *B. pertussis* but appears not to function similarly in *B. bronchiseptica*, although it is produced by most strains (Mattoo and Cherry 2005). The recently

identified BatB autotransporter plays a role in subversion of innate immune clearance mechanisms by *B. bronchiseptica* (Williams et al. 2008). Specific roles in virulence have been identified for only a few other autotransporters, primarily in *B. pertussis* (Mattoo and Cherry 2005; Wells et al. 2007). *B. avium* appears to possess seven intact autotransporter genes, none of which are orthologs to those of the classical *Bordetella* (Sebaihia et al. 2006). The function or importance of autotransporters other than pertactin and BatB in *Bordetella* of veterinary interest is largely unknown.

Adenylate Cyclase Toxin

The adenylate cyclase toxin (ACT) belongs to the "repeat in toxin" (RTX) family of bacterial pore-forming toxins, so named on the basis of conserved, calcium-binding glycine- and aspartate-rich repeats (Masin et al. 2006; Vojtova et al. 2006). It is a multifunctional, single polypeptide chain of 1706 amino acids with an N-terminal adenylate cyclase (AC) domain and a C-terminal hemolysin and pore-forming domain. The hemolysin may function independently of the AC domain, but both domains are required for AC activity. Internalization of ACT occurs through a receptor-independent mechanism (Martin et al. 2004) as well as a specific interaction with a β-2 integrin on the surface of many cells of the immune system. Unlike most RTXs, the majority of ACT remains associated with the bacterial cell surface, likely through interaction with FHA. However, it is the portion actively secreted into the external milieu that appears to be important for target cell intoxication. Yet, ACT does not function as a typical secreted toxin since intoxication additionally requires a close physical association between the bacterium and target cell. Once delivered to the eukaryotic cell cytosol, the AC domain is activated by the regulatory protein calmodulin. This results in unregulated conversion of cellular ATP to cyclic adenosine monophosphate (AMP), thereby disrupting cell signaling. Recently ACT was also demonstrated to elevate the level of calcium in target cells, potentially enhancing the activity of the toxin (Fiser et al. 2007).

ACT is essential for virulence and plays a critical role in infection by disabling innate immunoprotective functions, including phagocytosis, chemotaxis, and superoxide production, and by modulating immune responses through alteration of cytokine secretion. It also triggers apoptosis in macrophages, but cytotoxicity is likely effected through multiple pathways and depends on both pore-forming and AC activities (Hewlett et al. 2006). ACT has been suggested to contribute to colonization, but this is perhaps a manifestation of the functional stabilization of FHA rather than direct participation as an adhesin (Perez Vidakovics et al. 2006).

The structural gene for ACT, *cyaA*, is part of the *cyaABCDE* operon; CyaC catalyzes a posttranslational modification required for full activity, and cyaB, D, and E are required for secretion. ACT is produced by all classical *Bordetella* species, but each is antigenically distinct (Gueirard and Guiso 1993). Notwithstanding its demonstrated importance in virulence, the toxin appears nonessential for the success of *B. bronchiseptica* in certain niches based on the recent discovery of a unique phylogenetic lineage, hypovirulent in mice, missing the *cya* operon (Buboltz et al. 2008). Investigators have variously reported the absence (Gerlach et al. 2001) or presence (Donato et al. 2005) of *cyaA* and production of ACT in *B. hinzii*, a discrepancy likely due to interspecies differences (K.B. Register, unpublished data). No ACT-like activity has been detected in *B. avium*, and the *cyaA* operon is not present in the genome (Sebaihia et al. 2006).

Dermonecrotic Toxin

Dermonecrotic toxin (DNT) is an intracellular, heat-labile, ~160-kDa protein that causes necrotic lesions, splenic atrophy, or death, depending on the dose and route of administration, in a variety of laboratory animals (Hoffmann and Schmidt 2004; Masin et al. 2006). The level of DNT expressed by *B. bronchiseptica* has been correlated with the severity of nasal and lung lesions in experimentally infected pigs, and purified toxin impairs bone formation, likely through inhibition of osteoblast differentiation. Studies using *B. bronchiseptica* DNT mutants established unequivocally that the toxin is required for the induction of pneumonia and turbinate atrophy in both mice and pigs. In contrast, *B. pertussis* DNT appears to have a minimal role in whooping cough. DNT is produced by all members of the *B. bronchiseptica* cluster, and the amino acid sequences are highly conserved. *B. avium* also produces DNT and likewise requires it for virulence (Temple et al. 1998), although there is considerable divergence in the deduced amino acid sequence as compared with the DNT of other *Bordetella* species (Sebaihia et al. 2006). DNT mutants of *B. bronchiseptica* and *B. avium* display reduced ability to colonize the upper

respiratory tract (Temple et al. 1998; Brockmeier et al. 2002), but this is most likely an indirect effect related to a lesser degree of tissue damage rather than any adhesive property of the toxin. Sequence-dependent approaches suggest that DNT is absent in *B. hinzii* (Gerlach et al. 2001).

Delivery of DNT to target cells is initiated through binding of the N-terminus to an unknown receptor. The toxin is subsequently cleaved by a host cell protease, and the enzymatically active C-terminus is released into the cytoplasm, where it catalyzes modifications of regulatory guanosine triphosphate (GTP)-binding proteins, leading to aberrations in cytoskeletal organization, protein and DNA synthesis, and other processes important for homeostasis and normal cellular function.

Tracheal Cytotoxin

Initially characterized as a component of *B. pertussis* culture supernatants toxic for ciliated epithelial cells, tracheal cytotoxin (TCT) was subsequently determined to be a muramyl dipeptide fragment of peptidoglycan resulting from normal cell wall remodeling during growth in many gram-negative bacteria (Luker et al. 1995). Such fragments are typically transported from the periplasm back to the cytoplasm by the AmpG protein, where they are reused in the biosynthesis of newly forming peptidoglycan. AmpG is also expressed in *Bordetella*, but for reasons currently unclear, the vast majority of TCT is released extracellularly. TCT causes ciliostasis and extrusion of ciliated cells from the respiratory epithelium via induction of IL-1 in associated mucus-secreting cells and consequent accumulation of the free radical nitric oxide. TCT alone may elicit cytokine production from other cell types, but its effect on epithelial cells requires, and is synergistic with, endotoxins (Flak et al. 2000). *B. bronchiseptica*, *B. parapertussis*, and *B. avium* (Gentry-Weeks et al. 1988) also produce TCT.

Osteotoxin

A cytotoxic protein expressed by *B. avium*, distinct from DNT, was demonstrated to be lethal for cell lines derived from the trachea and bones (Gentry-Weeks et al. 1993). Originally referred to as an osteotoxin, cloning and sequencing of the gene and characterization of the purified protein revealed the gene to be an ortholog of *metC*, which encodes a β-cystathionase involved in the biosynthesis of methionine. Toxic effects appear to be due to reactive derivatives arising from β-cystathionase-catalyzed cleavage of extracellular L-cystine. The gene and corresponding toxin are also present in *B. pertussis* and *B. bronchiseptica*, but *metC* appears to be a pseudogene in *B. parapertussis* (Gentry-Weeks et al. 1993; Parkhill et al. 2003). A role in virulence has been postulated, but no studies to date have directly addressed this question for any *Bordetella* species.

Lipopolysaccharide

Bordetella lipopolysaccharide (LPS) is a highly immunogenic, major constituent of the outer cell membrane and an important bacterial defense against host immune responses including antibodies, complement, antimicrobial peptides, and surfactants (Preston and Maskell 2001; Pilione et al. 2004; Schaeffer et al. 2004; Goebel et al. 2008). In addition, it shapes both innate and adaptive immune responses through modulation of host TLR4-mediated signaling. *Bordetella* express species- and even strain-specific lipid A structures through variable patterns of fatty acid substitution (Caroff et al. 2002). Structural variation within the core oligosaccharide has also been described. The O-antigen polysaccharides of *B. bronchiseptica* and the human cluster of *B. parapertussis* are homopolymers of a diaminohexuronic acid in which the terminal residue is highly modified. Interestingly, these modifications vary between subsets of strains and may represent a form of antigenic diversity (Preston et al. 2006). The O-antigens of *B. hinzii* and *B. avium* are composed of one or two additional diaminouronic acids (Preston and Maskell 2001; Caroff and Karibian 2003). Numerous LPS biosynthetic loci have been identified in species of the *B. bronchiseptica* cluster, and the function of at least some genes has been directly demonstrated or may be reasonably inferred from sequence similarity (Preston and Maskell 2001; Parkhill et al. 2003). Variation occurring in these loci corresponds to altered LPS structures in some strains (e.g., *B. parapertussis* strains of ovine origin, Cummings et al. 2004; Brinig et al. 2006), suggesting that the full repertoire of *Bordetella* LPS structures has yet to be defined. Many orthologs for LPS biosynthesis genes have been found in *B. avium*, but variable gene content and order within specific loci and the lack of obvious orthologs for O-antigen biosynthetic genes suggest this species may utilize novel pathways for its LPS biosynthesis.

Pertussis Toxin

Pertussis toxin is an adenosine diphosphate (ADP)-ribosylating toxin that, *in vitro*, exerts a wide variety of biological effects related to interference with cell signaling pathways (Masin et al. 2006). *In vivo*, it inhibits neutrophil recruitment, resulting in leukocytosis and delayed antibody-mediated clearance. The toxin structural genes (*ptxA–ptxE*) and those required for its transport (*ptlA–ptlH*) are found in all members of the *B. bronchiseptica* cluster, but toxin expression has been observed only in *B. pertussis*.

Type III Secretion System

The type III secretion system (TTSS), a virulence factor of many bacterial pathogens, consists of a needle-like macromolecular complex through which specific effector proteins that interfere with normal cellular functions are delivered directly to eukaryotic cells. *B. bronchiseptica* and ovine strains of *B. parapertussis* possess a functional TTSS, primarily encoded by the *bsc* locus, that induces non-apoptotic cell death and alters the host immune response, the latter possibly contributing to persistence of infection (Abe et al. 2008). The *bsc* locus includes 30 genes that direct the synthesis of the secretion apparatus, potential effector proteins, translocons (which form the pores in eukaryotic cells through which effectors are transported), and other components of this highly structured system. The only *Bordetella* effector protein so far characterized is BopC or BteA, whose corresponding gene lies outside the *bsc* locus.

The TTSS genes are highly conserved but not expressed in the laboratory strains of *B. pertussis* and human-adapted *B. parapertussis* that have been investigated. However, the recent demonstration of TTSS activity in low-passage clinical isolates of *B. pertussis* (Fennelly et al. 2008) indicates this system may be more widely operative in *Bordetella* than originally believed. *B. avium* does not possess the *bsc* genes, and there is no evidence suggestive of a secretion system of this class (Sebaihia et al. 2006).

Iron Acquisition

Bordetella employ numerous mechanisms to overcome the severe iron limitation encountered in the host environment (Brickman et al. 2007). Members of the *B. bronchiseptica* cluster produce a dihydroxymate siderophore, alcaligen, that acquires iron from the eukaryotic iron-binding proteins lactoferrin and transferrin and is required for maximal virulence in *B. bronchiseptica* (Foster and Dyer 1993; Register et al. 2001). Species of the *B. bronchiseptica* cluster also utilize the catechol siderophore enterobactin, which they do not synthesize themselves, through a type of "siderophore piracy" wherein a *Bordetella* enterobactin receptor scavenges iron from enterobactin produced by other nearby bacteria. This receptor additionally sequesters iron from other structurally similar xenosiderophores, and its synthesis is up-regulated by certain host catecholamines, including epinephrine, norepinephrine, and dopamine, which may act as environmental cues to drive appropriate transcriptional responses. A homolog of the gene encoding the ferric enterobactin receptor has been identified and shown to be expressed in *B avium* (Sebaihia et al. 2006).

A system for heme utilization has also been characterized among species of the *B. bronchiseptica* cluster and in *B. avium*. Genome sequence analysis suggests that a number of additional iron-scavenging systems may be operative in *Bordetella*, with some unique to particular species (Parkhill et al. 2003; Sebaihia et al. 2006). The precise role of specific iron acquisition systems in the various *in vivo* and *ex vivo* environments encountered during the *Bordetella* life cycle currently remains unknown.

Biofilm Formation

Bacterial biofilms are surface-adherent communities of bacteria embedded within an extracellular matrix of bacterial proteins, DNA, and polysaccharides. Biofilms afford protection against host defenses and significantly increase resistance to antibiotics and environmental stresses. Growth of *Bordetella* within biofilms, characterized by production of a poly-β-1,6-GlcNAc encoded by the *bps* locus, has recently been demonstrated both *in vitro* and *in vivo* (Mishra et al. 2005; Irie and Yuk 2007; Sloan et al. 2007). The *bps* locus is required for biofilm formation and persistent colonization of the nasal cavity in mice by *B. bronchiseptica*. Few studies have so far addressed the complex process of biofilm development in *Bordetella*, but available data suggest that several adhesins, including FHA, fimbriae, and pertactin, also play a role in either formation or stability (Irie et al. 2006; Sloan et al. 2007; Serra et al. 2008). *B. bronchiseptica* growing in biofilms is highly tolerant of several antibiotics at levels toxic for planktonic cells (Mishra et al. 2005), and a biofilm mode of

existence may underlie the frequently chronic nature of *Bordetella* infection.

Genetic Regulation of Virulence Factors

Expression of all known protein toxins and adhesins of the classical *Bordetella* is positively regulated by a two-component sensory transduction system encoded by the *bvgAS* locus (Beier and Gross 2008). BvgS is a transmembrane protein with a periplasmic N-terminal domain responsive to environmental signals and a cytoplasmic C-terminal domain that interacts with the transcriptional activator BvgA. Typical *in vitro* growth conditions, including temperatures approximating 37°C and the relative absence of SO_4^- ions or nicotinic acid, activate a BvgS-dependent phosphorelay cascade, which ultimately leads to activation of BvgAS-inducible genes, which include *bvgA* and *bvgS* themselves, by phosphorylated BvgA (BvgA~P). Also among the BvgAS-inducible genes is *bvgR*, located immediately downstream of *bvgAS*, whose product acts to repress transcription from an alternate set of BvgAS-repressed genes. During growth at ~26°C or below or in the presence of high concentrations of SO_4^- ions or nicotinic acid, the activity of the phosphorelay cascade is greatly diminished, the level of BvgA~P falls, and transcription of BvgAS-induced genes ceases. Expression of BvgAS-repressed genes is simultaneously activated due to the absence of BvgR.

This process, termed phenotypic modulation, has until recently been envisioned as a reversible switch between two distinct phases: the Bvg^+ or virulent phase, characterized by maximal BvgAS-induced virulence gene expression, and the Bvg^- or avirulent phase, in which virulence gene expression is absent. Recent studies have led to the rediscovery of an additional, intermediate phase (Bvg^i; Cotter and Miller 1997) that was first described more than 50 years ago but largely forgotten until knowledge of the genetic basis for virulence gene expression in *Bordetella* began to emerge. The Bvg^i phase is triggered by semi-modulating conditions (e.g., low levels of nicotinic acid) and is characterized by expression of a unique set of genes not activated in either the Bvg^+ or Bvg^- phase as well as a subset of genes known to also be expressed in the Bvg^+ phase. It is now understood that promoters of BvgAS-regulated genes differ in their affinity for binding BvgA~P. Genes with high-affinity promoters, including those for adhesins such as FHA, are activated early in the Bvg^- to Bvg^+ transition by the relatively low levels of BvgA~P present during growth under semi-modulating conditions, and these genes are expressed in both the Bvg^i and Bvg^+ phases. Expression of "late" genes with low-affinity promoters, including those encoding many toxins, is triggered only by the high levels of BvgA~P that accumulate during growth in the fully modulated Bvg^+ phase. These and other observations have led to the current concept of phenotypic modulation in *Bordetella* as a rheostat rather than an on-off switch. Although only three phases have so far been documented (Bvg^+, Bvg^i, and Bvg^-), it is postulated that BvgAS controls a continuum of many different, possibly overlapping, expression states.

BvgAS controls expression of more than 500 genes, including those encoding known or putative virulence factors, metabolic enzymes, folding and transport proteins, and other functional categories. Maintenance of a system that precisely controls the transcription of such a large and diverse number of genes suggests phenotypic modulation is an important adaptive response in *Bordetella*, perhaps optimizing growth and survival in the various niches encountered within the host and during transmission. There is good evidence that the Bvg^- phase is neither expressed nor required for full virulence *in vivo* (Cotter and Miller 1994; Akerley et al. 1995), but it appears the switching mechanism itself must be operative for maximal colonization of the respiratory tract (Irie and Yuk 2007). Adhesins and other Bvg^i-phase gene products may assist in transmission and initial colonization of the host, while the late Bvg^+-phase virulence factors may be necessary for growth and immune evasion *in vivo*. Current data suggest that the Bvg^- phase, perhaps induced by low temperature, is optimal for the *ex vivo* environments encountered during transmission between hosts when there is no apparent need for toxins or other virulence factors. In *B. bronchiseptica*, motility is a prominent Bvg^--phase phenotype that may aid bacteria in the search for nutrients and environments more hospitable to growth. Interestingly, neither *B. pertussis* nor *B. parapertussis* expresses motility under any growth condition and, unlike *B. bronchiseptica*, neither appears able to survive for extended periods of time outside a host. However, the specific conditions that modulate gene expression in nature are unknown, and there is no definitive understanding of the role of modulation in the *Bordetella* life cycle.

The *bvgAS* locus is also subject to phase variation, as described for fimbriae, in which spontaneous deletions or frameshift mutations in the promoter irreversibly abolish expression of all *bvgAS*-activated genes. It is not known how, or whether, organisms in this state contribute to the success of *Bordetella* as pathogens.

PATHOGENESIS

Patterns of Disease

Bordetella of veterinary importance cause acute respiratory disease with high morbidity and low mortality but also circulate subclinically among susceptible populations (de Jong 2006; Ford 2006; Gaskell et al. 2006; Jackwood and Saif 2008). Infection occurs at any age, although young animals are at highest risk for acute disease, perhaps as a result of age-related alterations in the epithelium of the nasal mucosa (Larochelle and Martineau-Doize 1990) and/or immaturity of the immune system. *Bordetella* are frequently found in association with viral and other bacterial respiratory pathogens and predispose to secondary infection with certain of these agents. Such coinfections often result in increased severity of clinical signs; specific synergistic effects on colonization, pro-inflammatory cytokine production, and other aspects of disease have been demonstrated (Cook et al. 1991; Brockmeier et al. 2008). Temperature, humidity, air quality, animal density, and other environmental and management factors also affect the incidence and severity of disease. Common clinical signs include coughing, sneezing, dyspnea, oculonasal discharge, poor weight gain, and lethargy.

Despite its isolation from a wide range of host species, *B. bronchiseptica* is associated with clinically apparent disease in relatively few, including pigs (atrophic rhinitis and neonatal pneumonia), dogs (infectious tracheobronchitis), cats (tracheitis and bronchopneumonia), guinea pigs (bronchopneumonia), and rabbits (snuffles). A few genetically unique, closely related clones have been reported as the primary cause of death in several recent morbillivirus outbreaks in seals (Register et al. 2000). *B. avium* is a primary pathogen in domesticated turkeys (turkey coryza or bordetellosis) and an opportunistic disease agent in chickens. It is widespread among numerous wild avian species and causes clinically recognizable disease in at least a few. Until recently, there was no evidence that *B. hinzii* is capable of causing disease in hosts other than humans. It is now known that some strains induce mild to moderate respiratory disease in turkeys (Register and Kunkle 2009). *B. parapertussis* alone induces only mild respiratory disease in sheep, but nasal carriage is common, and infected lambs are predisposed to severe, acute *Mannheimia haemolytica* bronchopneumonia (Martin 1996).

Occasional episodes of zoonotic disease involving *B. bronchiseptica* or, more rarely, *B. hinzii* or *B. avium* occur, typically manifested as respiratory infection of young, elderly, or immunocompromised patients (Mattoo and Cherry 2005; Spilker et al. 2008; Harrington et al. 2009). Pleuritis, septicemia, meningitis, and wound infection have also been reported. In recent years, the frequency of reported *Bordetella* zoonoses has increased, including several cases involving immunocompetent individuals (Berkelman 2003; Rath et al. 2008). It is unclear whether this reflects a true rise in the number of cases or is the result of improved methods of identification.

The Disease Process

Bordetella are readily transmitted in natural host species through direct contact with infected animals or contaminated fomites. *B. bronchiseptica*, but not *B. avium*, is also spread via droplet aerosols (de Jong 2006; Jackwood and Saif 2008). The initial event in the disease process is colonization of the nasal cavity, which establishes a primary reservoir of infection for subsequent spread to the lower respiratory tract. The temperature at the surface of the nasal mucosa, particularly the anterior portion, is several degrees less than the internal body temperature, prompting the hypothesis that newly deposited *Bordetella* may exhibit temperature-induced "early" BvgAS$^+$- and Bvg^i-specific phenotypes, including production of FHA and fimbriae and FHA-dependent biofilm formation (Cotter and Miller 1997; Irie and Yuk 2007). Consistent with this are results of a recent *in vivo* study demonstrating FHA-dependent adherence of *B. bronchiseptica* to non-ciliated olfactory cells in the nasal cavity as well as formation of microcolony aggregates on the nasal mucosa (Irie and Yuk 2007). Nonetheless, definitive proof of the existence of Bvg^i-phase bacteria in the nasal cavity has yet to be presented.

Bordetella also readily attach to ciliated cells, and this is a prominent feature of infection in both the upper and lower respiratory tracts (fig. 21.1). The

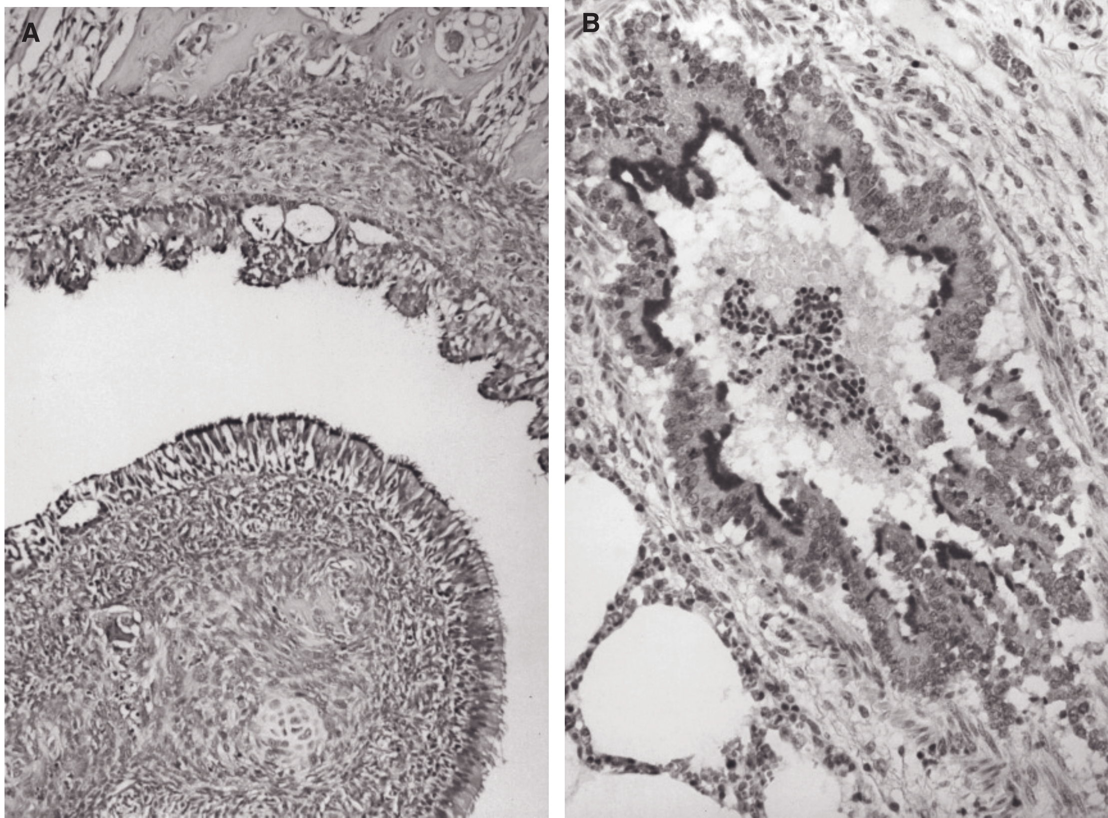

Figure 21.1. Colonization by *B. bronchiseptica* (red staining) of the ciliated epithelium of the conchae (A; 160×) or bronchus (B; 400×) of an infected pig. Areas denuded of cilia are apparent in the conchae. A neutrophilic inflammatory infiltrate is present in the lumen of the bronchus. (See color plate)

nature of this interaction in the nasal cavity is not understood, but it appears not to require FHA or fimbriae (Mattoo et al. 2000; Irie and Yuk 2007). In contrast, both *in vitro* and *in vivo* studies suggest a role for FHA, pertactin, fimbriae, and possibly additional adhesins in attachment to ciliated cells of the trachea (fig. 21.2; Cotter et al. 1998; Mattoo et al. 2000; Edwards et al. 2005). Replicating bacteria lead to production of TCT, likely responsible for initial impairment of the mucociliary clearance mechanism. Host cytokines and other inflammatory mediators elicited by the invading bacteria cause further tissue damage, thus opening the door to infection with other pathogens. As bacteria in the nasal cavity advance more deeply into the respiratory tract, the rise in temperature may promote a shift toward the Bvg^{+}-phase late gene expression, including synthesis of protein toxins that inhibit phagocytic cell function, further compromising microbial clearance. Although not true intracellular pathogens, *Bordetella* may invade and persist within phagocytic and other cell types (Brockmeier and Register 2000; Gueirard et al. 2005), which provide an environment protected from host immune responses.

Lesions

The severity of lesions in the nasal cavity in swine atrophic rhinitis varies widely depending on numerous factors, including the age of the animal, characteristics of the infecting strain, and housing and management (de Jong 2006). Atrophy of the ventral nasal turbinate bones is commonly seen at the height of infection but only rarely results in visible

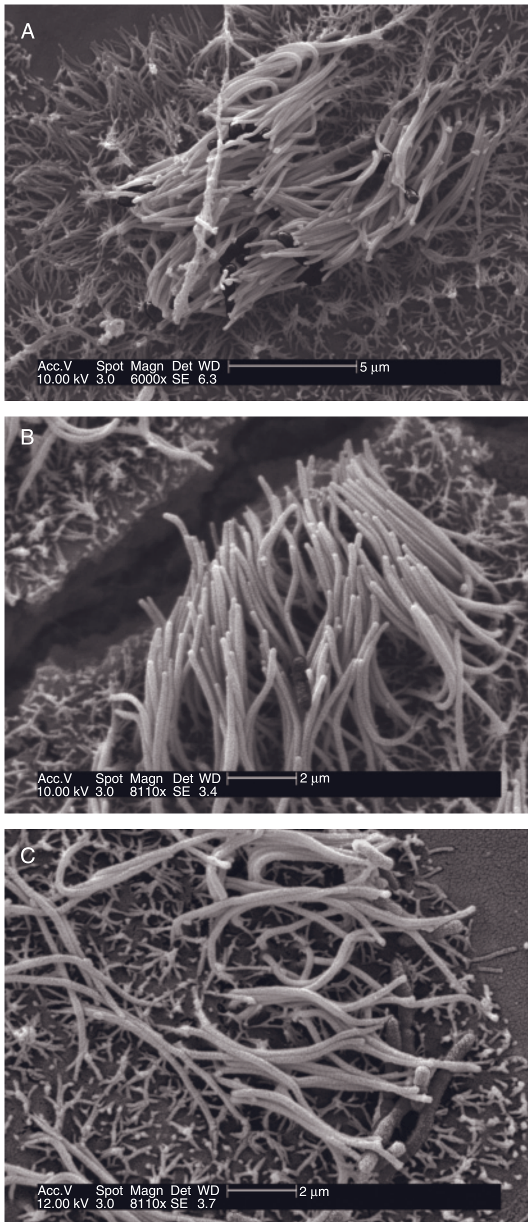

Figure 21.2. Binding of *B. bronchiseptica* to ciliated tracheal epithelial cells visualized by scanning electron microscopy. (A) Maximal binding, with preferential attachment to cilia, occurs when bacteria are in the fully modulated Bvg^{+} phase. (B) A mutant strain locked permanently in the Bvg^{-} phase displays a greatly diminished capacity for adherence. (C) Ectopic expression of FHA by a phase-locked Bvg^{-} mutant restores ciliary attachment. (See color plate)

shortening or distortion of the snout unless toxigenic *P. multocida* is also present. In uncomplicated *B. bronchiseptica* infection, there is complete or nearly complete regeneration of the turbinate, such that lesions are mild or undetectable at slaughter. Microscopically, attenuation of the epithelium and loss of cilia are apparent, accompanied by inflammatory cell infiltration and fibroblastic proliferation in the lamina propria. Similar lesions are evident in rabbits and rodents; tracheitis is an additional frequent finding in dogs and cats (Ford 2006; Gaskell et al. 2006).

Pneumonic lesions typically have a monolobular or bilobular distribution and are characterized by necrosis, alveolar hemorrhage, neutrophil accumulation, and, eventually, fibrosis. Bronchiolar obstruction by inflammatory cell infiltrate and mucopurulent exudate also occur (fig. 21.1B). The most prominent lesion of bordetellosis in poultry is softening and distortion of the tracheal rings, ultimately leading to tracheal collapse (Jackwood and Saif 2008).

Ultrastructural changes include loss of ciliated tracheal epithelium, multifocal infiltrates in the tracheal lamina propria, depletion of mucus from goblet cells, and multifocal lymphoid nodules in the submucosa. The bronchial mucosa is largely unaffected, although changes in the bronchus-associated lymphoid tissue and occasional heterophil infiltrates may be apparent.

Immunity

The immune response to *Bordetella* has been assessed in limited ways in naturally infected animals. Antibody titers are easily detectable, but cross-reactivity with other bacteria confounds interpretation. Substantial species-specific differences due to the diversity of strains and hosts, the complexities of other flora of the respiratory tract, and other parameters make it difficult to define the immune response in natural infections.

Experimental infections have been performed in a wide variety of animals, including pigs, rabbits, guinea pigs, rats, and mice. Rabbit infections with *B. bronchiseptica* strain RB50 showed that this isolate from a healthy rabbit has an infectious dose of less than 10 organisms (Mattoo and Cherry 2005). While bacteria were often observed in the lower respiratory tract, this was variable at different time points. However, all rabbits remained healthy but consistently infected in the upper respiratory tract for at least 6 months, suggesting that the sub-

stantial antibody response measured was not sufficient to completely clear infection. Guinea pigs and rats similarly infected with RB50 and other isolates from healthy rabbits followed a similar course as that described in rabbits; bacteria grew rapidly in the nasal cavity during the first week of infection, spread sporadically and probably transiently to the lower respiratory tract, and persisted indefinitely in the upper respiratory tract, usually without overt signs. Limited data with other *Bordetella* species are roughly consistent.

The immune response to infection has been examined primarily in mice because of the availability of immunological tools and genetically manipulated animals, as well as space and cost issues. The time course of infection was substantially the same in mice as in rabbits, rats, and other animals. Low-dose inoculations with *B. bronchiseptica*, mimicking natural infections, resulted in slow and sporadic infection of the lower respiratory tract, making it difficult to determine at a given time point whether bacteria had not yet spread to the lungs or had already been cleared. High doses, typically 10^5–10^6 CFU, can synchronously colonize all respiratory tract organs and challenge the immune response to reveal the critical aspects involved in protection from severe disease. The caveat is that some effects observed in these animals may be more reflective of relatively rare severe disease than the more common subclinical infections. However, a high dose inoculum induced substantial lung pathology, which allowed the contributions of various virulence factors to be directly measured. Additionally, since the animals remained apparently healthy, recovered fully, and cleared the infection from the lungs, the immune components involved in bacterial control and clearance could be dissected using the powerful tools of molecular immunology of the mouse model.

Innate Immunity

Bordetella bronchiseptica induces a robust highly inflammatory response to infection. *In vitro*, macrophages respond to *B. bronchiseptica* by increasing expression of many molecules that likely contribute to this inflammatory environment. A small subset of these molecules that are dependent on TLR4 for their expression, including TNFα, are apparently critical, since TLR4-deficient or TLR4-depleted mice are highly susceptible to infection with as few as 1000 CFU (Mann et al. 2004b). A very early peak in TNFα was also observed in the lungs of experimentally infected mice and is critical to early control of the infection; mice genetically deficient or depleted of TNFα with antibodies also rapidly succumbed to as few as 1000 CFU (Mann et al. 2004a). A host of other pro-inflammatory factors were induced and appeared to contribute to the highly inflammatory response to infection. However, certain cytokines, such as IL-10, appeared to modulate inflammation and the subsequent generation of adaptive immunity (Pilione and Harvill 2006).

Adaptive Immunity

Mice lacking B cells failed to clear *B. bronchiseptica* from the lower respiratory tract, although they contained it and limited numbers to approximately 10^6 CFU (Kirimanjeswara et al. 2003). The high titers of antibodies generated were sufficient, when adoptively transferred to naive hosts, for protection of the lower respiratory tract, although they had no effect in the nasal cavity. IgA, present in low quantities in serum, is required for reduction in bacterial numbers in the nasal cavity (Wolfe et al. 2007). Mice lacking T cells also failed to clear *B. bronchiseptica*. Although the simplest explanation for this effect is that T cells are required for efficient generation of protective antibodies, it is likely that the interactions are more complex. As documented with *B. pertussis*, different T cell subsets are likely to contribute to clearance, or interfere with it, in different ways. *B. bronchiseptica* appears to take advantage of the ability of dendritic cells to skew the immune response. Mice lacking IFN-γ are more susceptible to infection and clear bacteria from the lower respiratory tract more slowly than do wild type (Skinner et al. 2005). By a mechanism involving the TTSS, *B. bronchiseptica* augments the production of IL-10, decreases IFN-γ, and delays clearance. Mice genetically deficient in IL-10 produced higher levels of IFN-γ earlier in the infection and eliminated *B. bronchiseptica* from the lungs and trachea more rapidly than did wild-type mice. Together, these data provide an example of how *B. bronchiseptica* manipulates host immunity through the TTSS. It appears likely that some of the many other factors that *B. bronchiseptica* induces and secretes similarly manipulate immunity to contribute to infection, persistence, and transmission between a wide range of different animal hosts.

Vaccines

B. bronchiseptica vaccines include live attenuated strains or antigen extracts of killed bacteria, alone and in combination with those of other pathogens. Intranasal vaccines generally induce higher antibody titers but can cause nasal discharge in some animals and are of limited efficacy in those previously exposed, probably because an attenuated vaccine strain cannot outcompete a resident wild-type strain. Newly described engineered avirulent strains may have improved safety and may prevent infection by wild-type strains, providing herd immunity (Mann et al. 2007). In contrast, parenteral vaccines have been shown to increase serum antibody levels even in exposed animals but can cause localized irritation and may induce lower levels of secretory antibody. Vaccines have been shown to protect against *B. bronchiseptica* tracheobronchitis in puppies and neonatal bronchopneumonia in pigs. However, experimental infections suggest that, similar to *B. pertussis* vaccines in humans, parenteral *B. bronchiseptica* vaccines are likely to prevent severe disease but have little effect on infection rates, and therefore fail to provide herd immunity.

CONCLUSION

Bordetella are exquisitely adapted to colonization of the respiratory tract mucosa, often in the absence of overt disease. The apparent evolution of host-restricted species from a broad host range *B. bronchiseptica* progenitor, coupled with the availability of species-specific genome sequences and global transcriptional profiling, provides a highly informed framework for investigation of the basis for host specificity. Such studies have already identified novel potential colonization factors and vaccine immunogens currently being targeted for further analysis. Although rarely used to date, multidisciplinary approaches that include comparative genomics, experimental microbiology in natural host model systems, and mathematical modeling could additionally facilitate an understanding of the complex host–pathogen interactions that occur during various stages of infection and disease.

Continued development of faster and cheaper methods for large-scale DNA sequencing increasingly permit both inter- and intraspecies genome-level comparisons that expose fascinating aspects of the population structure and evolution of the *Bordetella*. The recent establishment of a publicly available *Bordetella* MLST database, funded by Wellcome Trust, will further aid such studies and also assist in revealing the frequency with which novel lineages emerge as well as the potential importance of immune competition and other factors.

An appreciation of the finely tuned, dynamic transcriptional response mediated by the BvgAS signal transduction system in *Bordetella* as a continuum, as opposed to the previously held notion of an "on-off switch," is an important conceptual advance that will undoubtedly lead to a more complete understanding of the *Bordetella* life cycle. Of special interest is the possible role in transmission played by bacterial products specific for the Bvg^{i} phase and perhaps other, yet-to-be identified functional phases along the the Bvg^{+}/Bvg^{-} continuum.

Despite many recent advances, much remains to be discovered as to how the multiplicity of adhesins, toxins, and other products employed by *Bordetella* species in their respective hosts function co-operatively in the establishment and maintenance of infection and the development of disease.

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22
Brucella

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INTRODUCTION

Brucellae are intracellular pathogens that localize in lymphoreticular tissues and primarily cause reproductive losses in natural hosts. In many parts of the world, brucellosis continues to be, or is reemerging as, a significant zoonosis. As with other intracellular pathogens, *Brucellae* utilize multiple molecular mechanisms to enhance pathogenesis *in vivo*.

CHARACTERISTICS AND SOURCES OF THE ORGANISMS

The genus *Brucella* encompasses a group of gram-negative bacteria that survive almost exclusively in infected hosts with preference for localization in intracellular compartments of phagocytic, reticulo-endothelial, and specialized epithelial cells. Based on microbial characteristics and host specificity, the *Brucella* genus has traditionally been characterized as having six species: *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella neotomae*, *Brucella ovis*, and *Brucella suis*. Genomic analysis indicates that *Brucella* strains comprise a single genetic "species" (Vizcaino et al. 2000), but the epidemiologic and diagnostic benefits of assigning *Brucella* strains to separate "nomenspecies" based on their distinctive phenotypic characteristics are readily apparent. Relatively recent isolations of new and phenotypically diverse *Brucella* strains from sea mammals, voles, and a prosthetic breast implant have been reported, and this will certainly expand the number of nomenspecies included in the genus *Brucella* (De et al. 2008).

Brucella abortus, *B. melitensis*, *B. neotomae*, *B. suis*, and marine isolates are designated as smooth strains because of their surface expression of the O polysaccharide on their lipopolysaccharide (LPS). *B. canis*, *B. ovis*, and some vaccine strains are designated as rough due to their lack of surface expression of the O polysaccharide. Most *Brucella* strains have two circular chromosomes encoding approximately 3.2 kb. The genome sequences of the *Brucella* nomenspecies display an average of >94% identity at the nucleotide levels (Vizcaino et al. 2000; Chain et al. 2005), with *B. abortus* and *B. melitensis* being the most closely related. A close relationship has also been detected between *B. canis* and *B. suis*, whereas *B. neotomae* and *B. ovis* demonstrate greater divergence levels from the other *Brucella* species. With the exception of biovar 5, *B. suis* isolates cluster together (Whatmore et al. 2007). Bacteriophages have been isolated from *Brucella* strains (Corbel 1987), but plasmids have not. The extent to which these bacteria are able to undergo genetic exchange among themselves or with other bacteria is unknown, and it has been proposed that intracellular niches inhabited by *Brucella* during residence in the host limit their opportunities for genetic exchange with other bacteria. The genetic homogeneity of *Brucella* strains and the close genetic relationships they maintain with other members of the α-proteobacteria, including the *Sinorhizobium* and *Agrobacterium* species (which infect plants), support this proposition. Traditionally, after isolation of gram-negative coccobacilli with cultural and biochemical characteristics consistent with identification as a *Brucella* spp., species

differentiation has been based on phage and dye sensitivity, CO_2 dependence for growth, production of H_2S, and serum agglutination tests. However, since microbial isolation of *Brucella* can be slow and tedious, other more rapid DNA-based methods have been developed for identification.

Some of the classical nomenspecies (*B. abortus*, *B. melitensis*, and *B. suis*) are divided into biovars based on biochemical, phenotype, and antigenic properties. Biotyping has been used for epidemiologic purposes, but it is somewhat subjective because it is based on subtle differences. Genetic mutations responsible for the biovar phenotypes remain to be determined (Whatmore et al. 2007).

Although *Brucella* strains have traditionally been given their nomenspecies designations based on their preferred host, all the pathogenic strains have the capability to infect other hosts. For example, under field conditions, *B. suis* and *B. melitensis* have been isolated from cattle and *B. abortus* from feral swine. Clinical symptoms of brucellosis are generally more severe in incidental hosts, including humans. At least four of the *Brucella* nomenspecies are pathogenic in humans, with *B. abortus*, *B. melitensis*, and *B. suis* considered to be highly pathogenic and *B. canis* to be less virulent. Clinical symptoms of recurrent fever, malaise, general debility, and hepatosplenomegaly are most common in human brucellosis, although symptoms may differ dependending upon the site of bacterial localization (i.e., meningitis and myocarditis). Although *B. canis* is considered less pathogenic, it can still induce typical clinical symptoms of brucellosis and more severe clinical syndromes if localized in aberrant locations. *B. ovis* and *B. neotomae* are not considered zoonotic. Zoonotic infections with marine *Brucella* strains have been documented as producing typical brucellosis symptoms. Although one marine *Brucella* strain did not cause disease when delivered conjunctivally to pregnant cattle (Rhyan et al. 2001); the limited data currently available would suggest that the virulence of these strains in aberrant hosts remains to be characterized.

Transmission

Members of the genus *Brucella* exist as intracellular pathogens in mammalian hosts. They do not exist as commensals nor are they found free-living in the environment (Roop et al. 2003). Although they can temporarily be recovered from environmental samples associated with infected animals, environmental persistence is generally accepted to be of no epidemiological importance since direct or close contact is required for transmission. Therefore, maintenance of brucellosis in an animal or a human population requires continual infection of susceptible hosts with *Brucella* spp.

Mechanisms of transmission for *Brucella* can generally be described by dividing the genus into virulent (*B. abortus*, *B. melitensis*) and low pathogenic (*B. ovis*, *B. canis*) strains. For the virulent strains, transmission primarily occurs through fluids or tissues associated with the birth or abortion of infected fetuses or to offspring through milk (Alton 1990; Thoen et al. 1993; Corbel 1997; Godfroid et al. 2004a, 2004b; Olsen and Stoffregen 2005). Since colonization in fetal fluids or placenta after *B. abortus* abortion can be as high as 10^9–10^{10} CFU/g and minimum infectious dosages are estimated to be in the 10^3–10^4 CFU range, abortion events can readily infect many animals with brucellosis. Venereal transmission is not considered to be important for transmission of *B. abortus* or *B. melitensis*. By contrast, venereal transmission is an important route of infection for *B. canis*, *B. ovis*, and *B. suis* (Godfroid et al. 2004b). *B. canis* can also be shed in urine, milk or from mucosal surfaces and has also been recovered from feces. Bitches may shed approximately 10^{10} CFU/ml in a serosanguinous vaginal discharge for 4–6 weeks after abortion. In general, animals infected with *B. canis* effectively shed *Brucella* for longer times from mucosal surfaces and urine when compared with animals infected with *B. abortus* or *B. melitensis* (Alton 1990).

B. suis strains are an exception to the categorical division described above because, although these strains are highly pathogenic in terms of the disease they produce in the host, they share transmission routes with both the virulent and low pathogenic strains of *Brucella*. Venereal transmission, for example, is important in the spread of *B. suis* infections in swine, but the bacterium can also be spread by contaminated milk, urine, and across mucosal surfaces. Like *B. canis*, infected swine effectively shed *B. suis* for long periods from mucosal surfaces. Although abortion storms have been reported with *B. suis*, data from experimental challenges suggest that it is more common for swine to maintain pregnancy for normal gestation periods as long as several fetuses remain alive. Experimental data suggest that shedding of *B. suis* can occur from the reproductive tract

even prior to parturition in infected sows (Stoffregen et al. 2006). The possibility of transmission of *B. suis* through consumption of dead fetuses or fetal membranes can also not be eliminated.

Brucellosis has been reported in several wildlife species, including bison, elk, wild boar, caribou, feral swine, and marine mammals (Godfroid 2004). Transmission between land mammals is similar to that reported for domestic animals (Olsen et al. 2004). The means by which *Brucella* are transmitted between marine mammals are unknown. However, the almost exclusive localization of these bacteria within the intestinal lumen and/or uterus of lungworms (*Parafilaroides*, *Phocoena*) in the pulmonary systems of an infected Pacific harbor seal and harbor porpoise suggests the intriguing possibility that parasites may play a role in disease transmission (Garner et al. 1997).

BACTERIAL VIRULENCE FACTORS

The capacity of *Brucella* to maintain long-term residence within macrophages serves as the basis for their ability to establish and maintain chronic infection (Roop et al. 2003). Within macrophages, *Brucella* remain enclosed in the phagosomal compartment. The extent to which *Brucella*-containing phagosomes mature along the endosomal–lysosomal pathway in host macrophages is an area of intense study. During long-term residence in the phagosomal compartment of host macrophages, *Brucella* are exposed to reactive oxygen intermediates (ROIs), acid pH, and nutrient deprivation (Roop et al. 2003). *Brucella* require acidification of the phagosomal compartment to a $pH < 4.5$ before they display wild-type intracellular replication. This requirement for low pH is transient and only extends through the initial stages of intracellular infection. Data suggest that low pH stimulates expression of genes, such as the VirB operon, which is required for successful intracellular adaptation. Several *Brucella* cell components have been linked to the resistance of these bacteria to exposure to low pH *in vitro*, but only CydB (a component of d-type cytochromes; Endley et al. 2001), Asp24 (a calcium binding protein of unknown function; Kahl-McDonagh and Ficht 2006), and urease (Bandara et al. 2007; Sangari et al. 2007) have been shown to contribute to the virulence of *Brucella* strains in experimental or natural hosts.

Bacteria with unlimited nutrients typically display exponential or balanced growth, whereas when nutrients become limiting, they enter a stationary phase (Roop et al. 2003). In the stationary phase, metabolism is primarily oriented toward maintaining cell viability over prolonged periods with little or no net increase in cell numbers. *Brucella* probably use both stationary and exponential stages for survival *in vivo*. Stationary-phase physiology provides *Brucella* potential benefits for successfully adapting to the harsh environmental conditions encountered in the phagosomal compartment. Evidence supporting the use of stationary physiology by *Brucella* includes the following: (1) During experimental infections in mice, *Brucella* tissue concentrations plateau quickly, with little or no replication demonstrated beyond the first 2 weeks post-infection; and (2) despite long-term antibiotic treatment of human brucellosis, relapses occur frequently, suggesting a stationary, non-replicative bacterial state (Roop et al. 2003). Molecular regulation of *Brucella* gene expression in the phagosomal compartment may differ as *Brucella* enter the stationary phase. VirB has been found to be maximally expressed during exponential growth, but its expression is repressed upon entry into the stationary phase (Roop et al. 2003).

One molecular mechanism that *Brucella* may utilize to survive nutrient limitations in the phagosome is *hfq* (host factor I). In other bacteria, production of *hfq* is required for translation of mRNA encoding the stationary-phase σ factor RpoS, which is required for efficient transition into stationary-phase physiology. *Brucella hfq* mutants demonstrate stationary physiology defects, including greater sensitivity to H_2O_2 and acidic pH, quicker loss of viability in minimal media, and accelerated clearance from the spleens of mice after the first week of infection (Roop et al. 2003).

Since oxidative killing is the primary mechanism employed by host phagocytes to control replication of intracellular pathogens, *Brucella* have multiple molecular mechanisms to detoxify free radicals. Expression of *sodC* is important for detoxification of O_2 radicals generated by the oxidative burst of macrophages. The *Brucella* gene *ahpC* is controlled by *hfq* and may also protect against oxidative killing. In other bacteria, *ahpC* protects against low levels of H_2O_2 and can detoxify peroxynitrite ($ONOO^-$), an oxidizing compound from macrophages believed to play an important bacteriocidal role. A *dps* gene,

a gene that plays a critical role in stationary-phase resistance to oxidative killing and acidic pH in other bacteria, has also been identified in *Brucella*. The LPS O chain also appears to protect against cellular cationic peptides and oxygen metabolites.

Effective acquisition of transition metals (Fe, Mn, Zn, and Mg) in the nutrient-restricted environment of the host is an important determinant of virulence. Transition metals are important for bacterial metabolism in the host. *Brucella* mutants that are unable to use high-affinity transport systems to capture Mn (E. S. Anderson, unpublished data), Zn (Kim et al. 2004; Yang et al. 2006), or Mg (Lavigne et al. 2005) are attenuated in mice. Studies with the mouse model also indicate that heme serves as an important iron source for *Brucella* strains in the host (Paulley et al. 2007). Several *Brucella* have been demonstrated to utilize heme as an iron source *in vitro* and in trophoblasts and macrophages (Roop et al. 2003; Cutler et al. 2005). *Brucella* scavenge iron through siderophores, such as 2,3 dihydoroxybenzoic acid or brucebactin (Bellaire et al. 2003), which are encoded by the *dhb*CEBA operon and regulated by iron response regulators and AraC-like transcriptional activators (Anderson et al. 2008). Mutation of iron regulatory genes has been demonstrated to attenuate virulent *Brucella* strains in mammalian hosts and make *Brucella* more sensitive to oxidative killing.

Successful adaptation to the differing environmental conditions *Brucella* strains encounter as they transit between hosts and extracellular and intracellular locations within the host requires programmatic changes in gene expression in these bacteria (Letesson and de Bolle 2004). *Brucella* have a quorum-sensing pathway involved in regulating the VirB type IV secretion system (Rambow-Larson et al. 2008) and a two-component system for environmental sensing of light (Swartz et al. 2007). BvrR and BvrS comprise a two-component regulator that controls the expression of genes encoding components of the cell envelope of *Brucella* strains (López-Goñi et al. 2002). *Brucella bvrRS* mutants are highly attenuated, indicating that cell envelope modifications carried out by BvrRS regulated genes play critical roles in interactions with the host. VjbR is a homolog of the LuxR-type regulators that recognize homoserine lactone-type quorum-sensing molecules in other gram-negative bacteria (Delrue et al. 2005). VjbR regulates expression of the genes encoding the type IV secretion machinery, and *vjbR* mutants are attenuated in experimental hosts. *Brucella* strains produce a C12 homoserine lactone (Taminiau et al. 2002), and this compound modulates VjbR activity *in vitro* (Uzureau et al. 2007), but exactly how "quorum sensing" by the brucellae contributes to virulence in the host has yet to be determined. Many bacteria employ small noncoding regulatory RNAs (sRNAs) to control the expression of genes at the posttranscriptional level (Gottesman 2004). This allows for rapid modulation of gene expression encoding gene products that are needed for quick adjustments to changing environmental conditions. The activity of the RNA chaperone Hfq is required for the vast majority of the bacterial sRNAs that have been examined. *Brucella hfq* mutants are highly attenuated in mice (Robertson and Roop 1999), goats (Roop et al. 2000), and nonhuman primates (Nikolich, unpublished data), suggesting that sRNAs play an important role in controlling the expression of the *Brucella* genes required for virulence in the host.

PATHOGENESIS

Overview

Brucella are intracellular pathogens that localize in lymphoreticular cells throughout the body and have a predilection for reproductive tissues. Upon entry through the plasma membrane, they modify the endosomal compartment of phagocytic cells to allow replication and long-term survival, use various mechanisms to modify the host environment, resist oxidative killing, and modify their metabolism to survive in their preferred intracellular environment. Cell-mediated immunity and Th1 cytokines, rather than humoral immunity, are important for protective immunity against brucellosis. Unknown molecular and/or physiologic mechanisms may contribute to species preference, recrudescence, and targeting of reproductive tissues in mammalian hosts.

Interaction between the Bacterium and Host Defenses

Brucella are highly adapted to survive within the hostile intracellular environment of monocytes. However, the host employs countermeasures that transform monocytes from permissive into resistant hosts capable of significant anti-*Brucella* activity. The most potent inducer of anti-*Brucella* activity is interferon-γ (IFN-γ). Stimulating monocytes/macrophages with IFN-γ triggers many antimicrobial activities, including increased production of

reactive oxygen (ROI) and nitrogen (RNI) radicals. However, in the case of *B. abortus*, ROI production was found to play a larger role in intracellular resistance to the bacteria than RNI (Jiang et al. 1993), whereas others have found that *B. suis*-infected monocytes reduced bacterial load primarily through the iNOS activity (Gross et al. 2005). Interestingly, pretreatment of monocytes with IFN-γ yielded the strongest anti-*Brucella* activity, which was characterized as a bacteriostatic mechanism that prevented intracellular replication rather than simply increased bacterial killing (Jiang et al. 1993). Recent discoveries in monocyte biology reveal that IFN-γ stimulates phagosome maturation and phagosome/lysosome fusion and increases rates of autophagy, which degrade intracellular vesicles and organelles. Under these conditions, it is difficult for even the most successful intracellular pathogens, such as *Mycobacterium tuberculosis* and *Leishmania*, to avoid phagosome/lysosome fusion.

In vivo or *in vitro* infections of mammalian hosts with *Brucella* is often associated with production of IFN-γ and Th1 cytokines, but human infections may switch predominantly to Th2 cytokines with chronicity (Rafiei et al. 2006). Data suggest the *virB* type IV secretion system is important for inducing IFN-γ and Th1 cytokine responses under *in vivo* conditions (Rolán and Tsolis 2008). Exogenous administration of IFN-γ or IL-12 to macrophage cell lines under *in vitro* conditions or *in vivo* to laboratory animals can reduce colonization with *Brucella*.

Like other intracellular pathogens, *Brucella* have adapted to their intracellular lifestyle and no longer require the accumulation of energy-storing molecules (Chain et al. 2005). The presence of cytochromes (cytochrome bc1 complex or quinol oxidase) with a high oxygen affinity may represent an important adaptation of *Brucella* to their intracellular lifestyle. As discussed, *Brucella* have a complete and functional type IV secretion system that is highly conserved at the nucleotide level. *Brucella* do not possess classic virulence factors, such as toxins, but rather those identified apparently facilitate cell invasion and survival by subverting innate cellular defense mechanisms.

Entry into Host Cells

Entry of non-opsonized smooth *Brucella* into cells relies on the cytoskeleton of the host cell for internalization. Smooth strains of *Brucella* interact with cholesterol-rich lipid rafts within the plasma membrane to facilitate contact with the host cell and mediate internalization by phagocytes and nonprofessional phagocytes. Lipid rafts, which contain glycosphingolipids, cholesterol, and glycosyl-phosphatidylinositol anchored proteins (Cutler et al. 2005), facilitate membrane-associated sorting events, such as the formation of multi-subunit membrane complexes, and signaling across membranes and membrane fusion. Lipid rafts are found in intracellular organelles, vesicles, and the plasma membrane. The *Brucella* LPS O polysaccharide appears to be a key molecule for interaction with lipid rafts on host cells but also prevents complement-mediated bacterial lysis and host cell apoptosis (Bagüés et al. 2004). Opsonization of smooth strains of *Brucella* increases entry tenfold; however, entry is targeted to the phagolysosomal compartment. Opsonization targets *Brucella* to specific receptors on the surface of phagocytes (Fc receptor for bound IgG, complement receptors for C3b and C4b) and diverts smooth bacteria from entry via lipid rafts. Receptor-mediated phagocytosis leads to greater killing of internalized *Brucella* by monocytes.

Entry of rough strains of *Brucella* differs from smooth strains. Because rough strains of *Brucella* are unable to sustain interactions with lipid rafts, they are readily phagocytosed following either Toll-like receptor 4 (TLR4) or mannose receptor recognition of the LPS-deficient bacterial surface. As a result, rough strains demonstrate elevated invasion possibly due to exposure of ligands that are normally hidden by the O chain and may have increased capability to adhere to macrophages (Bagüés et al. 2004). Consequently, non-opsonized rough *Brucella* are internalized as efficiently as opsonized *Brucella*, are rapidly targeted to the phagolysosomal compartment, and are generally unable to replicate (Rittig et al. 2003). Entry of smooth and rough *Brucella* strains into the cells through different pathways may also involve receptors with a distinct ability to regulate the level of phagocytosis. In contrast to rough strains that are defective at intracellular replication, the intracellular smooth *Brucella* surviving after opsonin-mediated phagocytosis are capable of significant intracellular replication (Bellaire et al. 2005). Genes necessary for O side chain synthesis (i.e., *manB*, *wboA*) play a significant role in establishing the intracellular replicative compartment for smooth strains of *Brucella*.

Intracellular Pathways Utilized

Internalized *Brucella* quickly traffic through the early endosomal compartment in host macrophages and depart the phagosome to form the modified phagosome ("brucellosome") by acquiring components of the endoplasmic reticulum (ER) in a manner similar to autophagosome biogenesis. The modifications of the phagosome prevent its fusion with lysosomes.

Brucella initially localize within acidified phagosomes (Rittig et al. 2001), where they are exposed to free oxygen radicals generated by the respiratory burst. Early localization in an acidified environment is important for *Brucella* replication and survival by inducing expression of the *VirB* operon (*virB* 1–10), an operon that controls expression of genes associated with a type IV secretion system. The VirB operon interacts with the ER in a way that neutralizes the pH of the phagosome (Celli 2006) and transports effector molecules, such as VceA and VceC (de Jong et al. 2008), into the cytoplasm of the infected macrophages to interfere with phagosomal maturation. The modified phagosome environment provides conditions of nutrient depletion and limited oxygen availability.

Another intracellular survival mechanism for *Brucella* involves modification of the lipid content of the phagosome-limiting membrane. Virulent *Brucella* strains express a cyclic glucan synthase (*cgs*) that produces and secretes low-molecular-weight cyclic glucans. These molecules disrupt the lipid raft microdomain structures within intracellular membranes surrounding the bacteria, a modification that inhibits phagosome maturation and prevents fusion with lysosomes and is independent from *VirB* and type IV secretion systems (Arellano-Reynoso et al. 2005).

Lesions and Disease Characteristics

Brucella primarily enter through penetration of mucous membranes, although infection through breaks in the epithelium is possible. Licking or sniffing aborted fetuses and placental membranes or ingesting contaminated milk introduces the *brucellae* to the oral and nasal mucosa, tonsils, and gastrointestinal mucosa. *Brucella* can penetrate the epithelium-covering domes of ileal Peyer patches. With *B. canis B. ovis*, and *B. suis*, entry can also occur through the mucous membranes of the reproductive tract.

Following penetration of mucosa, bacteria are transported, either free or within phagocytic cells, to regional lymph nodes, which become enlarged due to lymphatic and reticuloendothelial hyperplasia and inflammation. If bacteria do not become localized, and are not killed in regional lymph nodes draining the site of infection, they may replicate and spread to other lymphoreticular tissues and organs via the lymph and blood. With most *Brucella* strains, the bacteremia is short-lasting, such that live bacteria are not readily isolated from blood samples of infected individuals. Particularly with *B. canis* and to a lesser extent with *B. suis*, the bacteremia is longer in duration or occurs frequently during infection.

Localization in the reproductive or mammary glands is associated with the most severe pathology and capability to transmit infection. Hosts are more susceptible to infection with *Brucella* strains during pregnancy, although host mechanisms responsible for the increased susceptibility are not fully characterized. Infection with the brucellae during midgestation is associated with the greatest pathogenicity in cattle, although the probability of isolation of *B. abortus* at parturition increases as the days of gestation at the time of exposure increase (Crawford et al. 1987). *Brucella* gain access to the uterus and fetus via a hematogenous route, and the bacteria initially localize within erythrophagocytic trophoblasts of the placentome. Adjacent chorioallantoic trophoblasts become infected, leading to an inflammatory response. Infected trophoblasts may eventually rupture and ulcerate the chorioallantoic membrane. In addition to the hematogenous route, fetuses may also become infected via ingestion of amniotic fluid containing *Brucella*. Other than a diffuse submucosal inflammatory reaction, the endometrium is not infected with *Brucella* and remains largely intact.

The physical and hormonal characteristics of the placenta that allow immune suppression and prevent maternal immune-based rejection of the developing fetus likely play an important role in this susceptibility since the bulk of the bacterial replication that takes place during *Brucella* infections occurs within placental trophoblasts, host cells that are considered to be "immune privileged." In ruminants, the timing of maximum production of the four-carbon sugar alcohol erythritol by placental trophoblasts coincides with the period when these animals exhibit their greatest susceptibility to *Brucella* infections.

Erythritol is the preferred carbon source for *Brucella* strains, and it has been proposed that the presence of this compound is an important factor driving the extensive intracellular replication exhibited by the brucellae in placental trophoblasts during the latter trimester of pregnancy. Data suggest that mutations in erythritol uptake and metabolism genes contribute to the attenuation of *B. abortus* strains *in vivo* (Crasta et al. 2008). This postulated relationship between erythritol metabolism and the virulence of *Brucella* strains in ruminants has not been directly tested experimentally.

Brucella may also reside in a dormant, nonreplicative state in phagocytic cells. Calves infected with *B. abortus* at a young age often do not demonstrate seroconversion until after puberty. Recrudescence of clinical brucellosis is also a problem in human patients. Molecular mechanisms controlling recrudescence or host physiology influencing *in vivo* replication of *Brucella* have not been identified.

The most common clinical manifestation of brucellosis in natural hosts is reproductive loss through abortions, birth of weak offspring, or infertility. Other clinical signs associated with brucellosis in these hosts are relatively rare, but occasionally *Brucella* spp. can localize in joints, bones, or other aberrant locations producing inflammation and associated pathology. In contrast, *Brucella* infections in humans are frequently associated with clinical illness, including recurrent fever ("undulant fever"), general debility, hepatosplenomegaly, and other symptoms associated with inflammatory responses to infection.

Immunity

Although *Brucella* strains induce a vigorous immune response in infected animals, the chronic nature of the *Brucella* infections indicates that that these immune responses are insufficient to eliminate *Brucella* from their intracellular niche. Indeed, there is considerable evidence suggesting that a critical determinant in the pathogenesis of *Brucella* infections is the capacity of the bacteria to inhibit or avoid components of the host's immune defenses. Lipid A of the LPS of *Brucella* strains stimulates a greatly reduced inflammatory response in mammalian hosts than does the endotoxin of other gram-negative bacteria (Barquero-Calvo et al. 2007). This results in reduced infiltration of neutrophils and reduced cytokine production by infected animals during the early stages of infection, conceivably giving the brucellae a chance to reach their intracellular niches, where they would be protected from polymorphonuclear leukocytes (PMNs), complement, and antibodies. The unusual chemical composition of the O chain of the LPS of smooth *Brucella* strains also plays a direct role in inhibiting host immune responses. Specifically, macrophages have great difficulty in degrading the perosamine O chain of the *Brucella* LPS, and undigested O chain directly inhibits the ability of infected macrophages to present antigens to T cells via the major histocompatibility (MHC) class II pathway (Forestier et al. 2000).

Adaptive immunity, involving interactions between CD4+ and CD8+ T cells, macrophages, and dendritic cells are key to providing immunity against *Brucella*. The antigen-presenting cells (APCs) present antigens with MHC class I or II molecules to T cells, which undergo clonal expansion to develop responsive populations that can react to current and future infections with that pathogen (Wyckoff 2002). It is the αβ T-lymphocyte subsets that undergo antigen-specific clonal expansion in response to vaccination or infection and generally manifest specific immunity. In general, antigens derived from the outside and that enter the APC through phagocytosis for subsequent degradation in the phagolysosome are presented through the class II MHC, exogenous pathway. By contrast, antigens synthesized within the APC cytoplasm and transported to the ER are presented with class I MHC and processed through endogenous pathways. Recent work has suggested that pathogens such as *Listeria monocytogenes* and *Salmonella* spp. can lead to trafficking of proteins out of the phagolysosome and into the endogenous antigen pathway (Wyckoff 2002). The intracellular location or entry of antigens into the cell is important since processing via the exogenous pathway is typically associated with a Th2 response, which is predominantly humoral. However, antigens presented on the surface of the APCs via the endogenous pathway tend to evoke a Th1 response associated with cell-mediated immune responses.

Optimal immunity to intracellular bacteria, such as *Brucella*, is generally believed to be mediated by the Th1 subset of CD4+ lymphocytes and is associated with the production of IFN-γ and tumor necrosis factor α (TNF-α). Secretion of IL-12 and/or IL-18 by APCs works synergistically through different signaling pathways (STAT 4 and p65/

p50 NF-κB, respectively) to enhance the production of IFN-γ, TNF-α, and IL-2 from T cells (Tizard 2009). IFN-γ and TNF-α activate macrophages, leading to enhanced production of ROI and RNI by these phagocytes, and experimental studies have documented that activated macrophages have an increased capacity to kill intracellular brucellae compared with their unactived counterpartments.

Development of cytotoxic T lymphocytes may also play a role in protection. Following killing of infected cells by activated CD8+ T cells, intracellular bacteria within the cells may be released and be available for uptake and destruction by activated macrophages (Wyckoff 2002). Infected host cells killed by cell-mediated processes may also be killed by apoptosis and display signals that trigger phagocytosis and degradation without release of intracellular bacteria. Microarray data from infections of macrophages with smooth *Brucella* strains indicate that infection is associated with a down-regulation of genes associated with apoptosis (Rajashekara et al. 2006). Prevention of host cell apoptosis may be one mechanism used by *Brucella* to facilitate intracellular survival and persistence.

In general, antibodies are considered to play a minor role in long-term protection against brucellosis, although they may opsonize bacteria and facilitate phagocytosis by macrophages, leading to antigen processing/presentation and clonal expansion/activation of T cells. Entry of opsonized bacteria into phagocytes mediated by the Fc receptor also exposes these bacteria to a stronger oxidative burst. Stimuli that induce Th2 cytokines, such as interleukins 4 (IL-4) and 10 (IL-10), are aligned with humoral responses and do not lead to macrophage activation and protective immunity against *Brucella*. Inoculation with killed *Brucella* bacteria tends to induce primarily a non-protective Th2 response. This is most likely due to the processing of dead bacteria via an exogenous antigen presentation pathway.

The role of Toll-like receptors (TLRs) in an innate and adaptive immunity to microbes is a subject of considerable recent interest. Dendritic cells are of particular interest since they express TLRs that function *in vivo* as immunological sensors for the detection of pathogens. When pathogen-recognition receptors are triggered, the dendritic cells integrate the signals and migrate to the T cell areas of secondary lymphoid organs to stimulate naive T cells.

In mice, heat-killed *B. abortus* can stimulate dendritic cells to secrete both TNF-α and IL-12 (Huang et al. 2005). Dendritic cells secrete TNF-α after stimulation of the TLR4 pathway, whereas secretion of IL-12p40 is dependent on stimulation of the TLR9 and the MyD88 signal transduction molecule. The TLR4 pathway (fig. 22.1) may be of importance for bacterial pathogens since this TLR recognizes LPS as a ligand, can induce signaling through TLR-signaling adaptor proteins (MyD88, TRIF, TRAM, and MAL), and is capable of inducing potent immune responses (Takeda and Akira 2004). There is considerable evidence indicating that the lipid A component of the *Brucella* LPS has a reduced capacity to induce inflammatory responses compared with lipid A molecules from other gram-negative bacteria, such as the *Salmonella* spp., and this lower biological activity may stem from a reduced interaction of the *Brucella* lipid A with TLR4.

The TLR9 pathway may also be of importance in immunological responses to *Brucella*. TLR9 interacts with CpG DNA and has been shown to translocate from the ER to the endosome after cell activation. In mouse dendritic cells, IFN-γ secretion is TLR9-dependent and requires endosomal acidification, suggesting that interactions between TLR9 and heat-killed *B. abortus* occur at the endosome. In murine macrophages, unlike dendritic cells, secretion of IFN-γ is dependent on the MyD88 adaptor molecule but only partially dependent on TLR9 (Huang et al. 2005).

The TLR2 pathway may also contribute as *Brucella* lipoproteins (Omp16, Omp19) can interact with TLR2 and stimulate cytokine (TNF-α, IL-6, IL-10, IL-12) release from dendritic cells.

Activation of TLR pathways is not only regulated by the specificity of ligand binding to appropriate receptors but also by diverse regulatory mechanisms for adaptors (i.e., MyD88, TRIP, TRAM, MAL) within signaling pathways. The *B. melitensis*, *B. abortus*, and *B. suis* genomes encode a gene (TcpB) and corresponding protein (Btp1) that is homologous to the Toll/Interleukin-1 receptor (TIR) domains found in host cell adaptor proteins. The TcpB protein interferes with MyD88-dependent TLR signaling through TLR4 and TLR2 pathways and impairs activation and maturation of dendritic cells (Cirl et al. 2008; Salcedo et al. 2008).

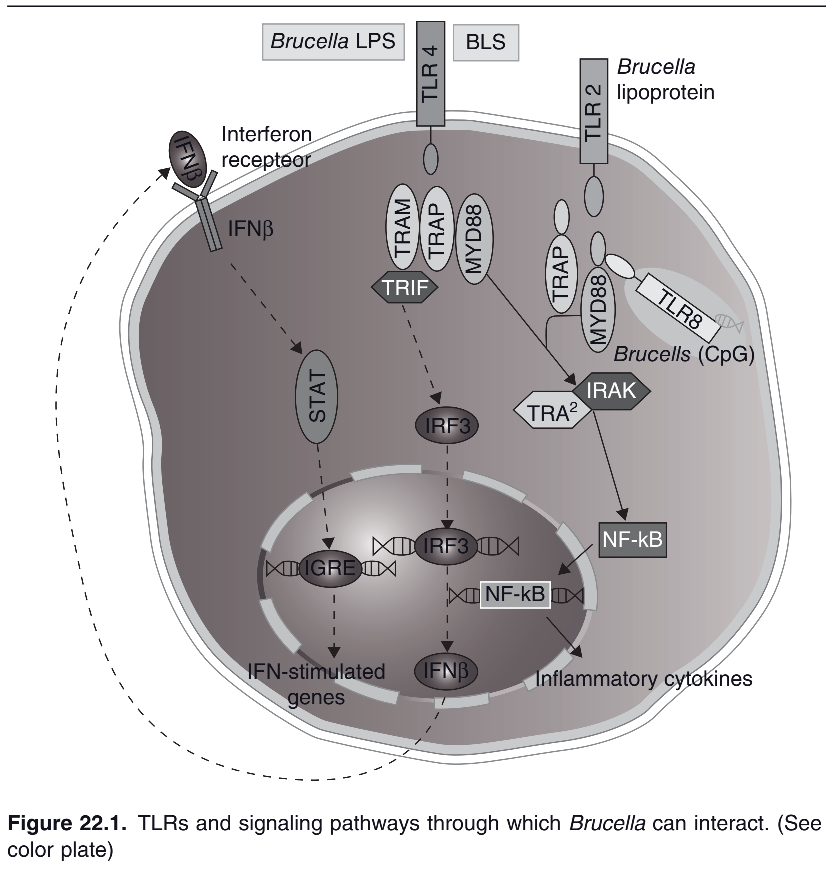

Figure 22.1. TLRs and signaling pathways through which *Brucella* can interact. (See color plate)

DISEASE CONTROL AND EPIDEMIOLOGY

Many countries have programs to control or eradicate highly pathogenic *brucellae* (*B. abortus*, *B. melitensis*, and *B. suis*). Although brucellosis can cause severe economic losses to livestock producers, most regulatory programs for brucellosis were developed to reduce brucellosis in animal reservoirs as a mechanism to prevent human infections. Multiple studies have demonstrated this approach to be the most cost-efficient mechanism for public health (Roth et al. 2003).

Regulatory programs can include sanitation programs to prevent disease transmission, vaccination programs to reduce herd susceptibility, and test-and-removal programs to eliminate animals infected with *Brucella*. Efficacious vaccines are available for *B. abortus* and *B. melitensis* but not for other *Brucella* spp. Although vaccination is highly effective in reducing production losses (i.e., abortions or weak offspring), it is less effective at preventing infection after exposure to field strains of *Brucella* spp. With efficacious vaccines, most vaccinated animals are probably only transiently infected but do develop antibody responses that react to brucellosis serologic tests used for surveillance. Although vaccination has been a very effective tool in controlling brucellosis, vaccination alone has never been effective in eradicating brucellosis. The most effective brucellosis programs incorporate combinations of sanitation, vaccination, and test-and-removal procedures.

Since brucellae are intracellular pathogens, microbial isolation is not always reliable, and most test-and-removal programs are based on removal of animals that demonstrate antibody responses to *Brucella*. Most serologic tests are based on the detection of antibodies against the O side chain of the *Brucella* LPS as it is a very immunodominant

antigen for humoral responses. Although the antibodies indicate that the individual's immune response has responded to infection with *Brucella*, it should be emphasized that seropositive responses do not necessarily indicate that the animal is currently infected or capable of transmitting brucellosis.

Factors accounting for differences between countries in brucellosis regulatory programs include prevalence of the disease within livestock or human populations, economic considerations, and the strength and prominence of livestock regulatory structures within the country. If there is a high prevalence of brucellosis within domestic livestock, regulatory programs may be designed to reduce prevalence rather than eradicate disease. Use of extensive test-and-removal programs in areas of high brucellosis prevalence may have unacceptable economic costs and may be devastating to livestock production in that area. In a similar manner, successful test-and-removal programs require that a country has strong regulatory and diagnostic structures for livestock disease control and sufficient resolve such that adequate numbers of livestock are being tested for brucellosis, livestock movement or change in ownership within the country is not occurring without brucellosis testing, and seroreactors are rapidly removed to prevent disease transmission. Since non-pasteurized dairy products are the primary mechanism for food-borne transmission, emphasis on testing of milk-producing livestock would be the most effective mechanism to prevent human infections that are not occupationally associated.

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Although progress has been made in characterizing the mechanisms that *Brucella* utilize for intracellular entry and persistence, there are many gaps regarding genes responsible for survival, regulatory control, and immunogenicity in the intracellular environment. It is anticipated that microarray techniques will provide valuable data on gene expression in *Brucella* that will address these knowledge gaps. Comparative studies of gene expression *in vivo* may also explain the observed phenotypic differences in host species preferences by different *Brucella* species.

Knowledge of specific genes or gene products that mediate protective immunity in various species is also lacking. A better definition of protective antigens may allow development of more efficacious vaccines for domestic livestock, which may include nonliving vaccines. In a similar manner, greater characterization of mechanisms by which brucellae subvert immunologic responses may also be beneficial for vaccine development. Increased knowledge in this area might allow development of a human vaccine, which could be beneficial for public health in areas with a high prevalence of brucellosis.

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23
Pseudomonas

E. L. Westman, J. M. Matewish, and J. S. Lam

INTRODUCTION

Pseudomonas are gram-negative rod-shaped bacteria that are motile by a single or multiple polar flagella. *Pseudomonas* species have remarkable nutritional diversity and can utilize a wide variety of organic compounds as carbon sources and electron donors for energy production. Four species of pathogens, namely, *Pseudomonas pseudomallei*, *Pseudomonas mallei* (responsible for melioidosis and glanders in animals, respectively), *Pseudomonas cepacia*, and *Pseudomonas maltophilia*, have been reclassified as *Burkholderia pseudomallei*, *Burkholderia mallei*, *Burkholderia cepacia*, and *Stenotrophomonous maltophilia*.

The most significant *Pseudomonas* species include *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas syringae*. *P. putida* and *P. syringae* are associated with plants, but only *P. syringae* is a plant pathogen. *P. fluorescens* is rarely pathogenic since it grows best at cooler temperatures, but it may contaminate blood products under refrigeration. *P. aeruginosa* is the predominant species that is associated with disease in humans and animals. Therefore, this chapter will focus on the significance of *P. aeruginosa* as a pathogen of animals, with reference to literature that has investigated *P. aeruginosa* pathogenesis primarily in humans.

Pseudomonas aeruginosa cells are ~0.3–0.5 µm by 1–2 µm in size and secrete the iron-sequestering siderophores pyoverdin and pyocyanin that give mature cultures a characteristic fluorescent-green or blue-green color, respectively (fig. 23.1). The optimal growth temperature is 37°C, but the bacterium can thrive at temperatures between 10 and 40°C. Although *P. aeruginosa* is classified as an obligate aerobe with a respiratory type of metabolism, growth can occur anaerobically using nitrate as an alternative electron acceptor. *P. aeruginosa* is ubiquitous in the environment and can be isolated from most natural habitats, including water, soil, plants, and sewage. It is often found in the hospital setting, including respiratory equipment, cleaning solutions, medicines, disinfectants, sinks, and floors, and accounts for about 10% of human nosocomial infections.

Differentiation of clinical isolates is important for epidemiological studies and is facilitated by numerous typing schemes. Serotyping is a commonly used serological method in clinical settings to classify *P. aeruginosa* based on differences in the O antigen of the lipopolysaccharide (LPS) present on the cell surface of these bacteria. Two forms of O antigens are produced by *P. aeruginosa*: the homopolymeric "A-band" O antigen, which is common to most strains, and the heteropolymeric "B-band" O antigen. Based on the saccharide composition and linkages of the B-band O antigen, the International Antigenic Typing Scheme (IATS) recognizes 20 serotypes of *P. aeruginosa*. Although the IATS is the most commonly used, other typing schemes have been employed in the past and include the Fisher immunotypes and the Lanyi and Bergan O serogroups. Other typing schemes for the classification of *P. aeruginosa* include biotyping, antibiogram analysis, pyocin typing, and bacteriophage typing, as well as more accurate and sensitive genotyping/DNA fingerprinting methods (which include pulsed-field gel electrophoresis and ribotyping) and

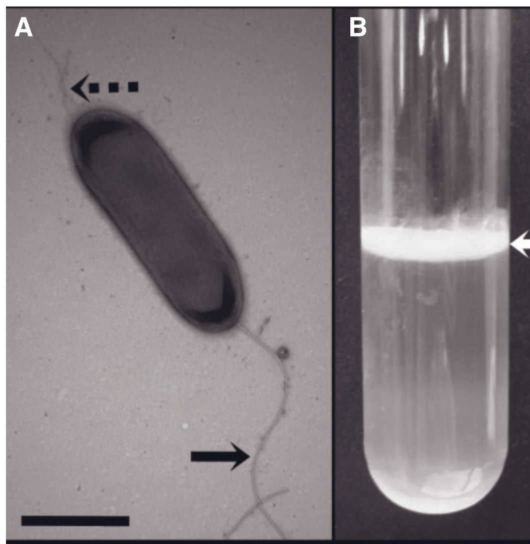

Figure 23.1. *P. aeruginosa.* (A) Transmission electron micrograph of negatively stained *P. aeruginosa* cells. Black arrow points to the single polar flagellum, and the dashed arrow indicates pili. Bar: 1 μm. (B) Stationary-phase culture normally exhibits the characteristic blue-green color due to siderophore secretion. The white arrow points to the pellicle, a biofilm at the liquid/air interface. (See color plate)

Table 23.1. Diseases in Animals Caused by *P. aeruginosa*

Animal species	Diseases
All species	Wound infection
Dogs, cats	Otitis externa, urinary tract infection, ulcerative keratitis, pneumonia
Birds	Respiratory infections
Cattle	Respiratory infections, mastitis, enteritis
Horses	Genital tract infection, abortion, ulcerative keratitis
Swine	Respiratory infections
Poultry	Septicemia, keratitis
Sheep	Pneumonia, mastitis, fleecerot, rhinitis, otitis
Mink	Hemorrhagic pneumonia, septicemia
Chinchillas	Pneumonia, septicemia
Laboratory rodents	Septicemia, enteritis

Source: Adapted from Gyles (1993).

polymerase chain reaction (PCR)-based techniques (such as amplified fragment length polymorphism analysis and random amplified polymorphic DNA analysis).

DISEASES

Pseudomonas aeruginosa is an opportunistic pathogen; it generally requires a defect or alteration in normal host defenses to establish an infection. Injury, trauma, surgery, burns, or indwelling devices, such as intravenous lines or urinary catheters, make the host susceptible to *P. aeruginosa* colonization. In humans, this pathogen can cause life-threatening infections in individuals who are compromised because of immune deficiencies and those suffering from burn wounds, cancer, cystic fibrosis (CF), and AIDS. The range of diseases includes pneumonia, bacteremia, and infections of the eye, urinary tract, and surgical sites. Treatment of infections by *P. aeruginosa* is often difficult due to the high intrinsic antibiotic resistance in this bacterial species (see the “Antibiotic Resistance” section). Generally, swift and decisive therapy is required to resolve the infection without promoting resistance to the antibiotic agent. Any animal suffering from a wound or trauma is a potential candidate for infection by *P. aeruginosa*, and other predisposing factors include chronic debilitating illnesses, prolonged hospitalization, prior therapy with antimicrobial drugs, and impaired host immunity (cellular and/or humoral) owing to a primary disease or as a consequence of immunosuppressive or cytotoxic therapy.

Pseudomonas aeruginosa causes a variety of diseases in animals, which include wound infections in all species, urinary tract infections and chronic purulent otitis externa in dogs, ulcerative keratitis in dogs and horses, and dermatitis (fleecerot) in sheep (table 23.1). *P. aeruginosa* infections in which there is no obvious breach in host defenses include mastitis in cattle, genital tract infections in horses, and rhinitis or otitis in sheep. In one case, several ewes developed severe purulent rhinitis and otitis externa/media after being showered with a wash that had previously been used on another group of ewes with dermatitis (Watson et al. 2003). Sporadic outbreaks of septicemic disease occur in poultry, mink, and chinchillas. Mink, in particular, are highly suscep-

tible to *P. aeruginosa* infection, as shown by an LD_{50} of 10^3–10^4 colony forming units observed in an intratracheal challenge, as compared with 10^6–10^9 in other species (Long et al. 1980). A vaccine has been developed to address severe hemorrhagic pneumonia in mink (Aoi et al. 1979). However, this vaccine has a limited range of protection for other animals and is not effective outside of mink and chinchillas.

One example of how the physiological versatility of *P. aeruginosa* contributes to this pathogen being an "opportunist" is clearly demonstrated in a study by Daly et al. (1999), who identified *P. aeruginosa* to be the causative agent of a severe outbreak of mastitis among 11 Irish dairy herds. It was a remarkable revelation that the teat wipes used on these dairy herds were contaminated with *P. aeruginosa* even though the wipes were stored in 70% alcohol. These wipes were used to sterilize the teats before injecting antibiotics into the mammary glands, and this provided a direct inoculation of *P. aeruginosa* onto the tissues.

The most commonly encountered *Pseudomonas* infection in animals is otitis in dogs, which typically presents with head shaking or ear scratching, purulent exudates, and malodor, as well as swelling, inflammation, and pain. Otitis externa and otitis media are often diagnosed together, even if the tympanic membrane is intact, and different bacterial species and/or different trends in antibiotic susceptibility may be found in the external and middle ears (Cole et al. 1998). In some otitis cases, initial therapy may fail or the otitis may reoccur rapidly following seemingly successful treatment. In these cases, culture and susceptibility testing are invaluable for selecting an appropriate antibiotic.

The success of *P. aeruginosa* as a pathogen is largely due to its ability to survive in a wide variety of environments and its arsenal of virulence factors (table 23.2). *P. aeruginosa* virulence factors can be divided into four groups: cell surface components, extracellular (secreted) products, type III secretion system (TTSS), and genetic regulatory

Table 23.2. Virulence Factors Produced by *P. aeruginosa*

Virulence factor	Proposed biologic effects
Cell-associated factors	
Pili	Adherence to epithelial cells, mucin, colonization of tissues
Flagellum	Chemotaxis, mediates motility for invasion, adherence to mucin
Lipopolysaccharide	Adherence and invasion of epithelial cells
O antigen	Serum resistance, resistance to phagocytosis
Lipid A	Endotoxic properties
Alginate/biofilm	Antiphagocytic, adherence to epithelium, antibiotic resistance
Extracellular products	
Exotoxin A	Cytotoxic, tissue invasion, cellular damage
Proteases:	
LasB and LasA elastase	Damage to lung tissue, blood vessels
Alkaline phosphatase	Tissue damage, increased vascular permeability, disruption of respiratory cilia
Type III secretion system (exoenzyme S, exoenzyme T, exoenzyme U, exoenzyme Y)	Cytotoxic, tissue damage and invasion, acute epithelial cell injury
Rhamnolipid	Ciliostatic, impairs mucociliary clearance
Phospholipase C	Destruction of pulmonary surfactant, generation of inflammatory mediators
Siderophores (pyochelin, pyocyanin, pyoveridin)	Acquisition of iron, enhances survival in low-iron environments such as host tissues

systems. Since this bacterial species is an important opportunistic pathogen of humans, our understanding of the pathogenic mechanisms contributed by virulence factors of *P. aeruginosa* is derived mostly from studies on animal models of human diseases.

OVERVIEW OF PATHOGENESIS

The pathogenesis of *P. aeruginosa* infections can be divided into three distinct stages: bacterial colonization, multiplication, and systemic disease (fig. 23.2). The first step in establishing an infection is colonization of host tissues. For an animal host, the first line of defense against bacterial invasion is the skin and the mucous membranes. However, if these tissues are damaged by injury or compromised by the insertion of a urinary catheter or intravenous line, bacteria will be able to attach to the epithelial cells predominantly by the cell-associated structures, such as pili, flagella, and LPS. Interestingly, damaged and regenerating respiratory epithelium express greater amounts of receptors on their cell surface for *P. aeruginosa* when compared with normal intact epithelial cells. For example, asialo-GM1, which is a glycosphingolipid receptor for pili-mediated adhesion of *P. aeruginosa*, is highly expressed on damaged epithelium (de Bentzmann et al. 1996).

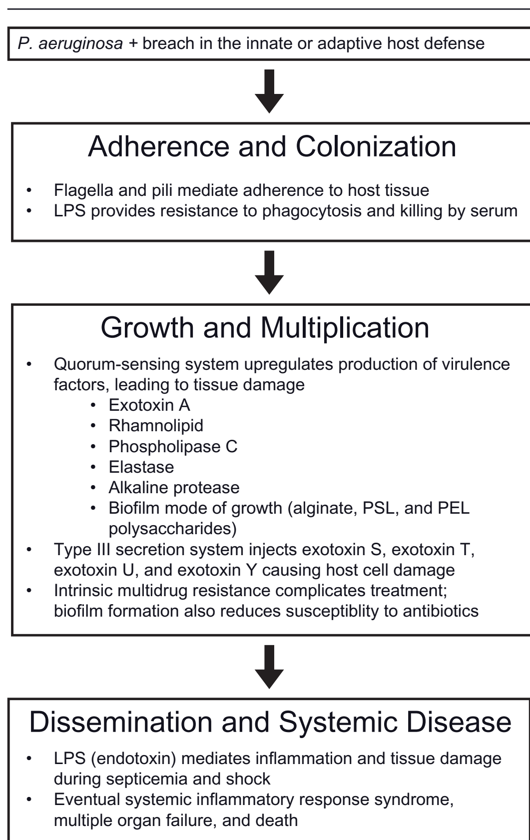

Figure 23.2. Overview of pathogenesis of infection due to *P. aeruginosa*.

Once colonization of tissues is established, bacteria begin to proliferate, and communication between *Pseudomonas* cells is attained through a cell-to-cell signaling system known as quorum sensing. Quorum sensing positively regulates the expression of a number of extracellular virulence factors, resulting in massive increase in the production of these excreted products (fig. 23.3). The extracellular products include exotoxin A (ExoA), LasA and LasB elastases, alkaline protease, rhamnolipids, and exoenzymes secreted by the TTSS. These factors are primarily responsible for tissue-damaging effects and further impairment of host defenses, thereby facilitating local tissue invasion. For example, the proteases of *P. aeruginosa* circumvent host defense mechanisms and mediate Hageman factor activation, immunoglobulin (Ig) and complement degradation, cytokine inactivation, and host protease activation. Iron is usually sequestered in the host. Survival of *P. aeruginosa* in the low-iron environment of the host tissues is facilitated by the production of siderophores, such as pyochelin and pyoverdin, which bind iron and transport it into the bacterial cell. Physiological changes also occur, leading to the expression of an exopolysaccharide called alginate and the formation of a complex community of surface-associated cells enclosed in a polymer matrix containing open water channels called a biofilm (Costerton et al. 1999; Donlan and Costerton 2002). Alginate also mediates attachment of mucoid *P. aeruginosa* cells to cilia of respiratory epithelial cells and to the glycoprotein-rich mucociliary blanket. Together, the multiple adhesion mechanisms improve the ability of the bacterial cells to colonize host tissues while the infection spreads. The overproduction of extracellular virulence factors, combined with the higher resistance to antibiotic and immune defenses because of the biofilm mode of growth, allows the bacteria to overwhelm host defenses.

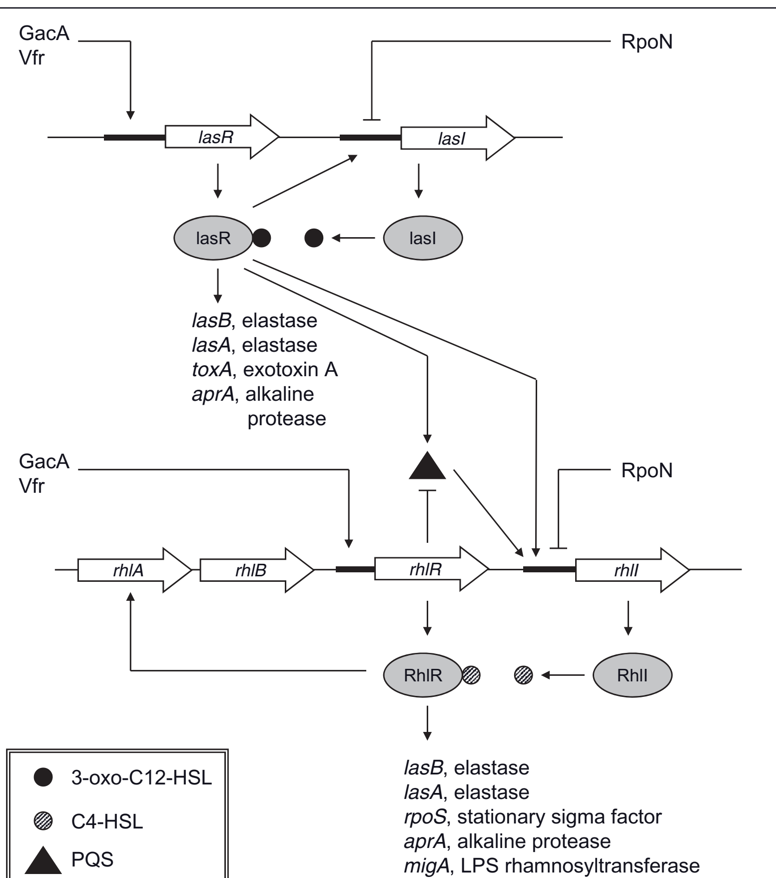

Figure 23.3. Quorum-sensing systems in *P. aeruginosa*. The cascade of events begins with the transcription of *lasR*, which regulates PQS production and the Rhl system, and ends with the activation of RhlR/C4-HSL controlled genes. Arrowheads indicate positive regulation, whereas a short parallel line indicates negative regulation. PQS, 2-heptyl-3-hydroxy-4-quinolone; 3-oxo-C12-HSL, *N*-[3-oxododecanoyl]-L-homoserine lactone; C4-HSL, *N*-butyrylhomoserine lactone.
Adapted from Venturi (2006) and from Van Delden and Iglewski (1998).

Dissemination of *P. aeruginosa* from the local site of infection, leading to systemic infection, is mediated by some of the same extracellular products responsible for local tissue damage. ExoA plays a significant role in systemic infections since a purified form of this toxin is highly lethal to animals and causes shock in dogs and rhesus monkeys (Pavlovskis et al. 1975). Patients with a high level of serum antibodies against ExoA at the onset of septicemia had a better survival rate than those with low antibody titers (Pollack and Young 1979). The lipid-A moiety of LPS, which is referred to as endotoxin, is also important in establishing systemic illness. Once the bacteria have invaded the bloodstream, host immune defense mechanisms generally exert bactericidal effects. However, the release of the endotoxin from the bacterial cell can result in excessive stimulation of the immune system and lead to fever, hypotension, oliguria, leukopenia, disseminated intravascular coagulation, and acute respiratory distress syndrome. Lipid A also activates clotting, fibrinolytic, kinin, and complement systems, stimulates the production of arachidonic acid metabolites (including prostaglandins and

leukotrienes), and induces the production of cytokines (including TNF-α). All of these factors implicate endotoxin in clinical syndromes associated with septicemia and have been reviewed by Alexander and Rietschel (2001). When compared with *Escherchia coli* in a canine model of septic shock, *P. aeruginosa* produced less endotoxemia but more cardiovascular dysfunction and mortality (Danner et al. 1990). These results reflect the multifactoral and complex nature of septic shock and suggest that bacterial products other than endotoxin and host-related factors may be important contributors to the toxicity, cardiovascular instability, and mortality of gram-negative septic shock.

As an understanding of the pathogenesis of *P. aeruginosa* evolves, the question arises as to whether different locations within the host influence the levels of various virulence factors to promote a distinct fingerprint of virulence factors for each disease. Hamood et al. (1996) examined human *P. aeruginosa* isolates from tracheal, urinary tract, and wound infections and concluded that "(1) elastase, phospholipase C, exotoxin A and exoenzyme S are produced by *P. aeruginosa* isolates from different sites of infection, (2) the production of higher levels of elastase and phospholipase C is important in all types of infections, while the production of exotoxin A and exoenzyme S is important in wound infections, and (3) persistent infection with *P. aeruginosa* may enhance exoenzyme S production." In a separate study, Lomholt et al. (2001) used a method called multilocus-enzyme electrophoresis to analyze clinical isolates to identify clones associated with disease. A *P. aeruginosa* clone pathogenic to the eye had a distinct combination of virulence factors characterized by high activity of elastase and alkaline protease, and possessed the gene for ExoU. Interestingly, *P. aeruginosa* isolates from canine otitis externa were found to possess an elastase-deficient phenotype (Petermann et al. 2001).

VIRULENCE FACTORS OF *PSEUDOMONAS AERUGINOSA*

Overview

The four groups of virulence factors differ in the role they play in the environmental life of an organism, the stage of pathogenesis influenced, and the target for manipulation by the factor. The cell-associated factors are generally thought to be essential for the viability of the organism even outside the host and include key structures in motility, adhesion, and the cell wall, as well as physiological states. The extracellular products are produced in order to gain nutrients or protect the cell from the immune response in the host, while the TTSS is a targeted mechanism for the delivery of several exotoxins directly into host cells. Finally, the regulatory systems enable *P. aeruginosa* to sense and react to environmental stimuli, such as the presence of other *P. aeruginosa* cells.

Integral Outer Cell Membrane Factors

Flagella

Pseudomonas aeruginosa produces a single polar flagellum that is required for swimming motility and is important for initial colonization of damaged or altered epithelium. This organelle allows the bacterium to propel itself and also functions as an adhesin to anchor the cell to the host epithelium. The flagellar filament is composed of flagellin proteins encoded by *fliC* and a cap protein encoded by *fliD*. Two distinct types of FliD proteins (types A and B) are involved in binding to secreted respiratory mucin glycoconjugates, and the type B cap protein specifically recognizes mucin bearing Lewis X or sialyl-Lewis X determinants (Scharfman et al. 2001). These determinants are often found on the surface of erythrocytes or other tissues of humans and animals. In addition, FliC proteins also bind to secreted mucin glycoconjugates bearing Lewis X determinants and to a cell-associated membrane-tethered mucin, Muc1, which is expressed by respiratory epithelial cells.

Using a neonatal mouse model of pneumonia, Feldman et al. (1998) demonstrated that *fliC* mutants of *P. aeruginosa* have decreased virulence. Histopathological examinations showed that the *fliC* mutant only caused focal inflammation, which is in contrast to the spread of inflammatory lesions throughout the lungs caused by the parent wild-type strain. Flagella mutants were less invasive than the motile wild-type strains in a burned-mouse infection model, and the addition of an anti-flagellum antibody had a significant protective effect (Montie et al. 1987). A bivalent *P. aeruginosa* flagella vaccine was recently evaluated in phase III trials in children with CF and was shown to elicit high and long-lasting levels of anti-flagellin serum IgG (Döring et al. 2007). Flagella are a target for non-opsonic bacterial clearance by macrophages and polymorphonuclear

neutrophils (PMNs) and may contribute to host clearance mechanisms by facilitating phagocytosis. Flagella also bind to epithelial glycolipid receptors, and this interaction stimulates the production of interleukin-8 (IL-8). IL-8 serves to target PMN migration and activates PMNs and macrophages. Thus, flagella may play a role in modulating the immune response during infection.

The flagellin monomers of *P. aeruginosa* are classified as a type or b type on the basis of molecular weight and reactivity with type-specific antisera. A-type flagellin (e.g, in *P. aeruginosa* strain PAK) is known to be glycosylated, and the glycan varies by strain (Brimer and Montie 1998; Schirm et al. 2004). More recently, the b-type flagellin of the *P. aeruginosa* strain PAO1 was also shown to be glycosylated (Verma et al. 2006). The role in pathogenesis of these flagellin glycans has not yet been studied in detail, although human anti-a-type flagellin monoclonal antibodies have been shown to react with deglycosylated flagellin (Brimer and Montie 1998), and loss of the glycan is not associated with changes in motility (Verma et al. 2006).

Pili

Pili are filamentous, polar cell surface structures of *P. aeruginosa* that are responsible for a form of flagella-independent motility, called twitching motility, which occurs over moist or semisolid surfaces. Pili are important for host colonization because they are thought to be the dominant adhesins of *P. aeruginosa*. The pili of *P. aeruginosa* are the prototypes of the type 4 family of pili, also known as *N*-methylphenylalanine pili. Cycles of extension, tethering, and retraction of the pili tow the bacterial cell forward, as if the cells were pulled along by a set of grappling hooks. Recent work has shown that pili extension and retraction are powered by the ATPase proteins PilB, PilT, and PilU (Chiang et al. 2008). Twitching motility is itself important for virulence because strains that were piliated but not able to twitch (due to deletion of *pilT* or *pilU*) were attenuated in virulence despite having the ability to associate with corneal epithelial cells as effectively as wild-type bacteria (Zolfaghar et al. 2003). In a recent study by Hasegawa et al. (2007), the authors performed an assay to test the ability of wild-type and non-piliated strains of *P. aeruginosa* to pass through membrane filters, and their data showed that pili were important for *P. aeruginosa* to infiltrate 0.22 μm filters.

A large number of genes are involved in the synthesis and function of the pili, but *pilA* could be considered the most important since it encodes the pilin monomer that makes up the fiber. The structure of a pilin monomer has been solved, and the oligomerization ability of PilA was found to be due to the interaction of the hydrophobic N-terminal region (Keizer et al. 2001). Each pilin monomer contains a functional receptor-binding site that is located in the C-terminal disulfide loop region. However, only the binding sites of monomers displayed at the pilus tip are functional. The receptor binding site of the pilin recognizes a disaccharide moiety, β-D-GalNAc(1→4)-β-D-Gal, which is found in many epithelial cell surface receptors. The major eukaryotic cell receptors that contain this disaccharide are the common cell-surface glycosphingolipids asialo-GM1 and asialo-GM2 and, to a lesser extent, lactosyl ceramide and ceramide trihexoside (Saiman and Prince 1993; Gupta et al. 1994). Pili also mediate adherence to many epithelial cell types *in vitro*, including human buccal cells, lung pneumocytes, exfoliating tracheal cells, immortalized human airway cells, and CF airway cells. In ulcerative keratitis, the binding of pili to the corneal cell receptor asialo-GM1 (Gupta et al. 1994) is essential for epithelial cell invasion and cytotoxicity (Comolli et al. 1999b). Pili also mediate adherence to DNA and abiotic surfaces, such as stainless steel, polystyrene, and polyvinylchloride.

Pilus-deficient strains are significantly reduced in their ability to cause epithelial cell damage, to persist at the site of infection, and to disseminate throughout the host (Woods et al. 1980; Sato et al. 1988; Tang et al. 1995). The assembly of pili is regulated by the two-component regulator system PilS–PilR and the sigma factor RpoN. Many other genes are also involved in pilus assembly, such as *pilJ*. Normal *P. aeruginosa* cells have an extended pilus at a single pole, but *pilJ* mutants produce truncated pili at both poles (DeLange et al. 2007).

Pili from some strains of *P. aeruginosa* are glycosylated. In strain 1244, the glycan is located at the C-terminus of PilA subunits, and the glycan was shown to be immunogenic, and anti-glycan sera were cross-reactive with LPS of the same strain (Comer et al. 2002). In strain PA5196, the glycan is a very unusual homopolymer of a rare sugar, D-arabinofuranose (Voisin et al. 2007). Fully glycosylated pilin is apparently important for virulence because a *pilO* mutant defective in the ligation of

O-glycan to the pilin protein became less capable of establishing an infection and surviving in the host when tested in a mouse respiratory infection model as compared with the wild-type bacteria (Smedley et al. 2005).

LPS

LPS of *P. aeruginosa* consists of a hydrophobic lipid-A component that anchors the LPS in the outer membrane, a core oligosaccharide that contains highly conserved sugar residues, and the long chain polysaccharide O antigen (also called O polysaccharide). LPS contributes significantly to virulence throughout the infection process, from initial attachment and colonization to avoidance of bacterial clearance during tissue invasion and dissemination in both acute and chronic infections.

Lipid A is composed of a phosphorylated diglucosamine backbone substituted with fatty acids and is the endotoxic moiety that mediates inflammation and tissue damage associated with gram-negative septicemia. When *P. aeruginosa* is killed by host factors, LPS from the cell wall is released into the tissues. In the bloodstream, circulating endotoxin is usually bound by the LPS-binding protein and transferred to the CD14 receptor and Toll-like receptor 4 on macrophages, inducing the production of inflammatory mediators including TNF-α, IL-1, IL-6, IL-8, and IL-10 (Lynn and Golenbock 1992).

The core oligosaccharide of *P. aeruginosa* is produced in two forms: an "uncapped" core oligosaccharide (lacking attached O antigen), referred to as "rough LPS," and a "capped" form with attached O antigen, which is referred to as "smooth" LPS (Sadovskaya et al. 1998). The structure of the core oligosaccharide is highly conserved among *P. aeruginosa* strains including clinical isolates. Evidence was obtained to show that bacteria possessing the rough form of LPS were particularly effective in adherence to and invasion of corneal epithelial cells in a cell culture assay. Similarly, rough LPS of *P. aeruginosa* was important in mouse infection models for the ability of *P. aeruginosa* to invade the eyes of the animals (Zaidi et al. 1996) and airway respiratory epithelial cells (Pier et al. 1996). Thus, *P. aeruginosa* LPS mediates internalization into epithelial cells. This is followed by replication so that a reservoir of intracellular bacteria is established and serves as a source to cause further spread of the infection. Epithelial cell receptors that recognize LPS as a ligand for attachment and invasion, found on both corneal and respiratory epithelial cells, include the cystic fibrosis transmembrane regulator (CFTR) protein, asialo-GM1, and galectin-3.

The O antigens that are attached to the capped form of core oligosaccharide are subdivided into two types: the common O polysaccharide, a repeating homopolymer composed of D-rhamnose; and the heteropolymeric O-specific antigen, which is composed of di- to pentasaccharide repeating units of a variety of unusual sugars that define the serotype. These structures have also been known as the A-band and B-band O antigens, respectively. Some of the rare sugars found in *P. aeruginosa* LPS are also present in other pathogenic bacteria, such as *Bordetella pertussis*, and similar biosynthetic strategies exist in both organisms (Westman et al. 2007, 2008). Numerous genes within the *wbp* locus are required for the biosynthesis and assembly of the O antigen (reviewed by Rocchetta et al. 1999). The O polysaccharides are antiphagocytic and confer resistance to complement-mediated killing. The O polysaccharide is required for virulence, as shown in a burned-mouse model study where the LD_{50} for the O antigen-deficient mutant was at least 1000-fold higher than that of the wild-type strain (Cryz et al. 1984). In a separate study, Tang et al. (1995) used a neonatal mouse model and demonstrated that an LPS mutant was unable to initiate a respiratory tract infection, whereas the wild-type strain caused acute pneumonia, bacteremia, and death.

Balance between the production of uncapped and capped form core oligosaccharides is governed by the expression of two putative rhamnosyltransferase genes, *migA* and *wapR* (Poon et al. 2008). The *migA* gene is involved in the production of uncapped core oligosaccharides (Poon et al. 2008), is highly expressed in respiratory tract infections (Wang et al. 1996), and is regulated by the quorum-sensing system (Yang et al. 2000). Thus, the role of the *migA* gene may account for the reduced amount of O polysaccharide seen in isolates from chronic infection (Yang et al. 2000).

Extracellular Products

Biofilm-Associated Polysaccharides

Biofilms are communities of surface-attached microorganisms enclosed in a matrix and play a significant role in infectious disease (Costerton et al. 1999). Biofilms are very resistant to the killing effect of whole blood and serum and affect the

pathogenesis of *P. aeruginosa* infections. The biofilm mode of growth also provides a two to three order of magnitude decrease in susceptibility to antimicrobial agents in comparison with planktonic (or free-swimming) cells. Interestingly, bacterial cells within a biofilm show reduced metabolic activities, making them less susceptible particularly to those antibiotics that target primarily metabolically active cells. The matrix, or extracellular polymeric substance, that holds the biofilm together can contain protein, polysaccharide, and nucleic acid. The polysaccharide components of *Pseudomonas* biofilms exocellular polysaccharide (EPS) are alginate, Psl, and Pel (reviewed by Ryder et al. 2007).

Alginate. *Pseudomonas aeruginosa* that are producing alginate are said to be "mucoid" because colonies have a distinctive slimy appearance. This slime-like exopolysaccharide is expressed under conditions of stress, such as low nutrients and high salt concentrations. Alginate is a linear copolymer of D-mannuronic acid and L-guluronic acid and is secreted into the extracellular environment after the bacterial cells have attached to their substratum. In the environment, alginate production confers protection from predation by flagellate protozoans during the early stages of biofilm formation. During infections, the secreted exopolysaccharide forms a viscous gel around the bacteria and can interfere with host immune defenses by acting as a direct physical and chemical barrier against phagocytic cells, interfering with both non-opsonic and opsonic phagocytosis, quenching reactive oxygen intermediates, scavenging hypochlorite generated by phagocytic cells, and stimulating the production of proinflammatory cytokines, including IL-1, TNF-α, and IL-8 (Govan and Deretic 1996). Mucoid strains are most often isolated from the sputum of human patients with CF, and microcolonies of *P. aeruginosa* have been observed in the lungs of these patients (Lam et al. 1980). Exopolysaccharide-enclosed microcolonies attached to bladder epithelial cells from catheretized patients have also been observed (Marrie et al. 1980). The mechanism for the overproduction of alginate is often a mutation in the *mucA* gene, which is a negative regulator of alginate production. Despite the importance of alginate to the resistance properties of biofilms, it is not required for biofilm formation, and other polysaccharides have recently been shown to be involved (Wozniak et al. 2003).

Psl. Nonmucoid strains of *P. aeruginosa* (those that express the negative regulator, *mucA*) produce little to no alginate but can still form biofilms, and these biofilms contain polysaccharide material (Wozniak et al. 2003). This material is produced by the polysaccharide synthesis locus (*psl*), which encode biosynthetic machinery essential for biofilm formation in *P. aeruginosa* PAO1 (Friedman and Kolter 2004b; Jackson et al. 2004). *psl* is required for cell surface and cell–cell interactions and functions as a scaffold to maintain the biofilm structure post-attachment (Ma et al. 2006). The carbohydrate composition and regulation of *psl* are not known and will require future research.

Pellicle. *Pseudomonas aeruginosa* is capable of forming a biofilm at the air–liquid interface of standing cultures, and these structures are called pellicles (fig. 23.1B). Production of a pellicle (Pel) is dependent on the *pel* locus, which encodes an operon required for the production of a glucose-rich matrix polymer (Friedman and Kolter 2004a, 2004b). Pel is also required for the maintenance of the biofilm structure in *P. aeruginosa* PA14 and PAK (Friedman and Kolter 2004a). The Pel polymer is biochemically and genetically distinct from Psl, but its structure is unknown. The Pel polysaccharide has also been implicated in initial attachment, but this effect can be masked if wild-type pili are present (Vasseur et al. 2005).

ExoA

ExoA, encoded by *toxA*, belongs to the family of adenosine diphosphate (ADP)-ribosyltransferase enzymes (like the diptheria toxin) and exerts cytotoxic activity on a wide variety of mammalian cells. ExoA is apparently the most potent virulence factor of *P. aeruginosa*, as determined by lethality in animals. For example, the LD_{50} of ExoA in mice is 0.2 μg/animal by the intraperitoneal route (Liu 1973) and 0.062 μg by the intravenous route (Callahan 1976). ExoA induces cell death in many mammalian cell lines, which is characteristic of apoptosis (Morimoto and Bonavida 1992) and necrosis (Plotkowski et al. 2002). Local tissue damage, bacterial invasion, and possible immunosuppresion in the infected host are largely attributable to ExoA. *In vivo* studies have shown ExoA to be critical for ocular damage, corneal ulceration, persistence of infection, and cell death in a mouse model of bacterial keratitis (Pillar and Hobden 2002).

ExoA affects the host defense system by inhibiting proliferation of human granulocyte and macrophage progenitor cells, altering the production of TNF-α by human leukocytes (Staugas et al. 1992), and interfering with murine IL-1 production by peritoneal macrophages (Misfeldt et al. 1990). ExoA is toxic to murine and human PMNs and inhibits phagocytosis and killing of *P. aeruginosa* by PMNs *in vitro* (Miyazaki et al. 1995).

ExoA is secreted in response to iron starvation and is under the regulatory control of the *lasR–lasI* quorum-sensing system. After secretion, ExoA binds to eukaryotic cells by the α 2-macroglobulin receptor and becomes internalized. Once internalized, the enzymatic subunit is translocated to the cytosol, where it transfers ADP-ribose from nicotinamide adenine dinucleotide (NAD) to elongation factor-2 (EF-2) and inhibits protein synthesis, resulting in cell death (Iglewski and Kabat 1975). The cytotoxic pathways of ExoA have recently been reviewed by Wolf and Elsasser-Beile (2008).

Rhamnolipid

Pseudomonas aeruginosa secretes a heat-stable glycolipid called rhamnolipid, which acts as a surfactant. Rhamnolipid has hemolytic activity and at high doses produces generalized cell membrane damage. Macrophages are lysed within minutes when incubated with as little as 6 µg/ml of purified rhamnolipids, and a variety of animal cell types are lysed within seconds of exposure to 100 µg/ml (McClure and Schiller 1992). At sublethal doses, rhamnolipids produced cellular distortion of macrophages and inhibited their ability to bind and/or ingest preopsonized *P. aeruginosa*.

The toxicity of rhamnolipid compared with other toxins of *P. aeruginosa* is relatively low (Liu 1974). Nevertheless, rhamnolipids play a role in the degeneration of lipids and lecithin, which may contribute to tissue invasion and necrosis. In respiratory tract infections caused by *P. aeruginosa*, rhamnolipids presumably solubilize the phospholipids in lung surfactant, making it more susceptible to degradation by other enzymes, such as phospholipase C (Liu 1974). The loss of the lung surfactant may contribute to atelectasis associated with both acute and chronic *P. aeruginosa* lung infections (Liu 1979). Rhamnolipids also impair normal host respiratory clearance mechanisms by slowing down mucociliary clearance (Read et al. 1992).

Two forms of rhamnolipids are produced: one contains one L-rhamnose sugar and the other contains two L-rhamnose sugars and both are linked to β-hydroxy-decanoic acid. These are called monorhamnolipid and dirhamnolipid, respectively. The biosynthesis of both forms has been reviewed by Soberón-Chavez et al. (2005). In brief, monorhamnolipid synthesis is catalyzed by a rhamnosyltransferase system, RhlAB, and dirhamnolipid production also requires RhlC, a second rhamnosyltransferase. Both *rhlAB* and *rhlC* are strictly regulated by the *rhl* quorum-sensing system, and production of rhamnolipids is restricted to high bacterial densities of cells grown under various growth conditions, including nitrogen or phosphate limitation.

Phospholipase C

Pseudomonas aeruginosa produces one or more homologous hemolytic phospholipases C (PLC). The best studied PLC is encoded by *plcH*, hydrolyzes phosphatidylcholine and sphingomyelin, and causes tissue necrosis and cell death *in vivo* (Coutinho et al. 1988). The other known *plc* genes are *plcN* and *plcB*. PlcN has a similar range of activity to PlcH, but only PlcB is active on phosphatidylethanolamine (Barker et al. 2004). Lung surfactant found in the bronchial alveolar tissues contains approximately 70% phosphatidylcholine, and *plcH* is induced in phosphatidycholine-rich environments by an AraC-family transcription factor (Wargo et al. 2008). Hydrolysis of phosphatidylcholine by PlcH produces phosphorylcholine and diacylglycerol, which may have toxic effects on the animal host by inducing the production of biologically potent substances (e.g., arachidonic acid metabolites and protein kinase C, which are known to alter eukaryotic cell metabolism and induce abnormal release of inflammatory mediators). PlcH is toxic to animals at microgram amounts and causes vascular permeability, hepatonecrosis, renal tubular necrosis, end organ damage, and death in mice at high doses (Berk et al. 1987; Meyers et al. 1992). Intradermal injection of sheep with PlcH causes superficial inflammatory lesions characteristic of fleecerot (Chin and Watts 1988). *plcH* mutants are less virulent in burned-mouse models of sepsis (Ostroff et al. 1989) and produce less lung injury and dissemination than the wild-type strain in a rabbit model of pneumonia (Wiener-Kronish et al. 1993).

Proteases: Elastase and Alkaline Protease

Elastase (LasB or pseudolysin) is a metalloprotease that has both proteolytic and elastase activities (Galloway 1991). Although its proteolytic activity exceeds its elastase activity, elastolysis is more important for pathogenesis because many tissues, such as the lung and blood vessels, contain elastin, which is required for their physiological function of expansion and contraction. Subcutaneous and intramuscular injections of LasB into mice cause severe hemorrhage and leakage of plasma components, including red and white blood cells, into the extravascular tissue (Komori et al. 2001). LasB is also capable of degrading transferrin, TNF-α, IL-2, components of the complement cascade, IgG, IgA, secretory IgA, and interferon-γ from T cells and receptors on neutrophils (Galloway 1991). Elastase is relatively nontoxic, with an LD_{50} for mice in the range of 60–400 μg, depending on the route of administration. Purified elastase, however, can damage intact pulmonary and corneal tissues (Gray and Kreger 1975, 1979) and can destroy epithelial cell junctions (Azghani et al. 2000). LasA or staphylolysin is a serine-type protease that nicks elastin, making it more susceptible to the elastase activity of LasB. The *las* and *rhl* quorum-sensing systems regulate expression of both *lasB* and *lasA* (Toder et al. 1991).

Alkaline protease or aeruginolysin, encoded by *aprA*, is a zinc metalloprotease that causes tissue damage and alters host defense mechanisms. It degrades a variety of substrates, including laminin, fibrin and fibrinogen, lactoferrin and transferrin, complement components C1q and C3, and proinflammitory cytokines IFN-γ and TNF-α (Parmely et al. 1990). *In situ* and *in vivo* experiments have shown AprA to increase vascular permeability and to disrupt the function of normal respiratory cilia. AprA contributed to virulence in a burned-mouse model of infection (Holder and Haidaris 1979). Early studies demonstrated that AprA is an important virulence factor associated with tissue damage in *P. aeruginosa* corneal infections (Howe and Iglewski 1984). However, results from a more recent study contradicted these findings. Pillar et al. (2000) reported that a genetically defined *aprA* mutant is equally as effective as the parent strain in virulence and in adherence to corneal tissues.

The TTSS: ExoS, ExoT, ExoU, and ExoY

The TTSS is a specialized protein complex that acts as a "molecular syringe" to deliver cytotoxins directly from the bacterium into the cytoplasm of eukaryotic cells. Over 20 genes are required for secretion, translocation, and regulation of this system. The secretion of four cytotoxic proteins, exoenzyme S (ExoS), ExoT, ExoU, and ExoY, is induced by direct contact between the bacteria and the host cells. These proteins are postulated to promote epithelial cell injury, inhibit the phagocytic response to infection, and allow bacterial replication. The PcrV protein is part of a channel-forming complex that is required for translocation of the effector proteins into the host cells. PcrV is required for virulence, lung injury, and cytotoxicity in an acute lung infection model (Sawa et al. 1999).

The best example of an effector protein is exoenzyme S (ExoS), a 49-kDa bifunctional cytotoxin that has two independent catalytic activities. The carboxy-terminus domain inactivates guanosine triphosphate (GTP)-binding proteins of the H- and K-Ras families and the cytoskeletal protein vimentin (Coburn et al. 1989; Coburn and Gill 1991), and the amino-terminus domain activates small-molecular-weight GTPases of the Rho subfamily and stimulates actin reorganization, which results in disruption of actin microfilaments (Goehring et al. 1999). Since the Rho and Ras proteins of eukaryotic cells control wound healing and tissue regeneration, ExoS interferes with the normal function of these proteins and contributes to the establishment of infection and chronic disease by *P. aeruginosa* by maintaining sites of colonization. ExoS causes cell injury and reduces proliferation and viability of host cells *in vitro* and is important for tissue damage and bacterial dissemination in animal studies (Nicas and Iglewski 1985; Kudoh et al. 1994). Inhibition of the Ras and Rho proteins may also interfere with both innate and acquired immunities, as both types of proteins are required for phagocytosis and Ras is required in T cell activation.

Exoenzyme T (ExoT) is a GTPase-activating protein for Rho GTPase proteins and interferes with Rho transduction pathways, which regulate actin organization, exocytosis, phagocytosis, and cell cycle progression (Krall et al. 2000; Sundin et al. 2001). Specifically, ExoT has been shown to target host kinases CrkI and CrkII, which regulate focal adhesion and phagocytosis (Sun and Barbieri 2003). Secretion of ExoT into the cytosol of the eukaryotic cell inhibits bacterial internalization by eukaryotic cells *in vitro* (Garrity-Ryan et al. 2000); however, the role of inhibition of invasion by ExoT in the

pathogenesis of *P. aeruginosa* infection is not understood. Recently, ExoT was shown to be necessary and sufficient to induce apoptosis in epithelial cells (Shafikhani et al. 2008).

The third effector protein, exoenzyme U (ExoU), is a lipase that requires activation or modification by eukaryotic factors (Sato et al. 2003) and is cytotoxic to a variety of mammalian cell types *in vitro* (Hauser et al. 1998). ExoU and ExoS are apparently mutually exclusive, as no strains expressing both have been isolated, and those strains that do express ExoU are more cytotoxic. Expression of ExoU is associated with lung injury and fatal infections in animal models (Allewelt et al. 2000), and cell death is characteristic of necrosis rather than apoptosis or oncosis (Hauser and Engel 1999). A reduction in the viability of infected murine macrophage-like cells and pulmonary microvascular endothelia cells was associated with the expression of ExoU and ExoS but not ExoT, ExoY, or PcrV (Stepinska and Trafny 2008).

The fourth effector protein, exoenzyme Y (ExoY), is an adenylate cyclase that induces elevation of intracellular cyclic adenosine monophosphate (AMP) *in vitro* and *in vivo* (Yahr et al. 1998), and its role in pathogenesis is unknown. Comparison of ExoS, ExoT, and ExoY knockout mutants suggests that ExoY may provide a minor contribution to systemic spread *in vivo* (Vance et al. 2005).

Regulatory Systems

Quorum Sensing

Quorum sensing in bacteria is a cell-to-cell signaling system that microorganisms use to communicate with each other in a cell density-dependent manner. It is used to modulate a variety of physiological processes, including the production of many virulence factors (reviewed in Parsek and Greenberg 2000). *P. aeruginosa* has two quorum-sensing systems, *lasR–lasI* and *rhlR–rhlI* (fig. 23.3). Each system is composed of two components: the transcriptional activator (LasR and RhlR, respectively) and an autoinducer synthase (LasI and RhlI, respectively). LasI and RhlI are involved in the synthesis of the cell-to-cell signaling autoinducer molecules *N*-[3-oxododecanoyl]-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyrylhomoserine lactone (C4-HSL), respectively. As a population of bacteria grows, the cell density increases and synthesis of the autoinducer molecules is upregulated. The autoinducers are released into the immediate area and move into the surrounding bacterial cells. At a certain threshold concentration, the autoinducers bind to their respective transcriptional activator proteins, LasR and/or RhlR. The autoinducer/transcriptional activator complex binds to conserved DNA sequences upstream of target genes, thereby upregulating their expression.

The regulation of quorum sensing is highly complex and has been reviewed by Venturi (2006). At the top of the regulatory hierarchy are the global regulators, GacA and Vfr. GacA is a transcriptional activator, and Vfr is a cyclic AMP receptor protein. Although the environmental stimuli that these global regulators respond to is not known, GacA and Vfr control quorum sensing at the level of transcription of the *las* and *rhl* systems (Albus et al. 1997; Reimmann et al. 1997). The *las* system controls expression of the virulence factors *lasB* (elastase), *lasA* (elastase), *exoA* (ExoA), and *aprA* (alkaline protease) and activates expression of *rhlI* and *rhlR*, the second quorum-sensing system. The *rhl* system regulates production of rhamnolipid and the stress response regulator RpoS and is also required for optimal expression of LasB elastase, LasA protease, hydrogen cyanide, alkaline protease, and the siderophore pyocyanin. Siderophores assist in the acquisition of iron, thus enhancing survival in low-iron environments and also give cultures of *P. aeruginosa* their characteristic color (fig. 23.1B). A more detailed review of these interrelated regulatory systems has been published by Miller and Bassler (2001).

Quorum sensing is essential for the pathogenesis of *P. aeruginosa* infections. This is substantiated by evidence that *lasR*, *lasI*, and *rhlI* mutants have reduced virulence when compared with the wild-type strain in many different animal models of infection (Smith and Iglewski 2003). In addition, the *las* cell–cell signaling system is required for normal biofilm formation, and *lasI* and *rhlI* mutants produce abnormal biofilms that are susceptible to biocides. The importance of the autoinducer molecules to the activation of many virulence systems has led to the investigation of autoinducer degradation as a possible therapeutic technique.

GacS–GacA

GacS and GacA are members of a two-component regulator system where *gacS* encodes the transmembrane sensor and *gacA* encodes the response regulator. GacA positively regulates the quorum-sensing

systems by upregulating the production of *N*-butyryl-L-homoserine lactone, RhlR, and LasR (Reimmann et al. 1997). Therefore, in a defined hierarchical manner, GacA indirectly regulates the expression of the virulence factors controlled by the *las* and *rhl* systems. GacA is essential for biofilm growth as a *gacA* mutant showed a tenfold reduction in biofilm formation relative to that of the wild-type strain. Furthermore, *gacA* mutant biofilms show a moderate increase in sensitivity to a range of antibiotics (Parkins et al. 2001). In a burned-mouse model, 77% of the animals inoculated with the wild-type strain succumbed to the infection in 36–48 h, but no mortality was observed in the animals infected with the *gacA* mutant (Rahme et al. 2000).

RpoN

rpoN encodes a sigma factor that enables RNA polymerase to recognize RpoN-specific DNA consensus sequences within promoter regions of target genes in response to various environmental stimuli. Thus, RpoN regulates the expression of a diverse set of genes required for growth as well as virulence, including genes required for biosynthesis of pili, flagella, alginate, and other regulatory genes (such as *rhlI*). RpoN has also been implicated in *P. aeruginosa* resistance to quinolones and carbapenems (Viducic et al. 2007). A *rpoN* mutant is less virulent in several mouse infection models, including a burned-mouse model (Hendrickson et al. 2001), a murine corneal scratch model (Preston et al. 1995), and an acute pneumonia model (Comolli et al. 1999a). In addition, *P. aeruginosa rpoN* mutants have a reduced ability to colonize tissues, as demonstrated in a chronic murine intestinal mucosal model (Pier et al. 1992).

Interconnections

As an opportunistic pathogen, *P. aeruginosa* is adapted to survival both in the environment and in the host. The extensive cross-talk and interconnections between production of seemingly disparate virulence factors probably reflects this diverse lifestyle. Some virulence factors are related through common biosynthesis steps, while others are coordinately or negatively regulated. The GacS–GacA and quorum-sensing systems explain much of this cross-talk, but other examples are not well understood.

For example, the quorum-sensing system regulates *migA* (Yang et al. 2000), which is a rhamnosyltransferase involved in LPS biosynthesis. Balance between the production of uncapped and capped form core oligosaccharides is governed by the expression of two putative rhamnosyltransferases, *wapR* and *migA* (Poon et al. 2008). Thus, the quorum-sensing system indirectly regulates the production of LPS glycoforms.

LPS biosynthesis shares biosynthesis steps with the flagellar glycan. In the *P. aeruginosa* strains PAK and PAO1, synthesis of the flagellar glycan requires genes involved in LPS biosynthesis (Miller et al. 2008; Lindhout et al. 2009). Although flagellin produced by certain LPS mutants is not glycosylated, flagella remained functional, but deficiencies in motility were observed and attributed to altered physicochemical properties on the cell surface, caused by changes in the LPS composition (Lindhout et al. 2009).

Many examples of coordinate or negative coregulation of virulence factors affect the flagella. Flagellar synthesis, bacterial motility, and adhesion to mucin are regulated by a two-component regulatory system involving two proteins, FleS and FleR. Expression of the cap protein FliD requires an additional transcriptional regulator FleQ. Recently, FleQ was also shown to be a cyclic-dimeric guanosine monophosphate (c-di-GMP)-responsive transcriptional regulator that represses transcription of genes, including the *pel* operon involved in exopolysaccharide biosynthesis (Hickman and Harwood 2008). Cross-talk between flagella and the TTSS has also been demonstrated, as strains lacking flagellar filament protein FliC exhibited a hyperefficent TTSS (Soscia et al. 2007). GacA is a positive regulator for motility, but strains lacking GacA showed hypersecretion of exotoxin S through the TTSS (Soscia et al. 2007). Flagella synthesis is also impacted by the production of alginate, which is controlled by a complex regulatory hierarchy (Wozniak and Ohman 1994). Binding of the intracellular second messenger bis(3′-5′)-c-di-GMP to the membrane protein Alg44 is also required for alginate biosynthesis (Merighi et al. 2007). MucR specifically regulates alginate biosynthesis by activation of alginate production through generation of a localized c-di-GMP pool in the vicinity of Alg44 (Hay et al. 2009). However, outer membrane protein profile analysis showed that overproduction of MucR mediates a strong reduction in the copy number of FliC, the main flagellin subunit (Hay et al. 2009).

Much of the cross-talk between virulence factors can be rationalized when thinking about the different stages of pathogenesis by *P. aeruginosa*. In the adherence and colonization phase, flagella and pili must be expressed to mediate attachment to the host tissue. LPS is also important for this phase because it gives resistance to killing by serum and to phagocytosis. Thus, sharing of biosynthetic intermediates between LPS and the flagellar glycan makes sense because both occur in the same phase. After the cells are established, they begin to multiply and accumulate autoinducer molecules, which activate the quorum-sensing system. The biofilm lifestyle is sedentary, so the flagella are no longer required. Thus, the coordinate repression of FliC with activation of alginate production by MucR is reasonable. These examples also underscore the vast physiological differences between planktonic and biofilm cells.

ANTIBIOTIC RESISTANCE

Pseudomonas aeruginosa is highly resistant to most antibiotics and disinfectants because of its physiology: intrinsic multidrug resistance is conferred by the low permeability of the outer membrane, presence of multidrug efflux pumps, production of β-lactamases, and other acquired resistance mechanisms. This organism is intrinsically resistant to a broad range of antibiotics, including penicillin, ampicillin, tetracycline, first- and second-generation cephalosporins, sulfonamides, neomycin, streptomycin, kanamycin, chloramphenicol, nitrofurans, and trimethoprim-sulfonamide. Only a few antibiotics are generally effective against *P. aeruginosa*, and these include fluoroquinolones, amikacin, gentamicin, certain broad-spectrum β-lactam antibiotics (such as imipenem and carbapenem), and a fourth generation cephalosporin, cefepime.

Outer Membrane Permeability

The overall outer membrane permeability of *P. aeruginosa* is 12- to 100-fold lower than that of *E. coli*, so the first challenge for successful antibiotic treatment is the entry of the agent into the cell. The outer membrane contains LPS molecules that are cross-linked by the binding of divalent cations, such as Mg^{2+} or Ca^{2+}, to negatively charged functional groups in the core, thus stabilizing the membrane. Polycationic antibiotics, such as gentamicin, tobramycin, and colistin, induce self-promoted uptake into the bacteria by competitively interacting with the negatively charged LPS functional groups. Polycationic antibiotics are much larger than the metal ions, and by binding to the LPS, these drugs disrupt the normal permeability barrier of the outer membrane to promote "self-entry" into the bacteria. A similar strategy of uptake applies to antimicrobial cationic peptides, which are part of the innate immune system of complex organisms and can possess direct antimicrobial activity and/or the ability to modulate innate immunity (Jenssen et al. 2006). When grown in divalent cation-limited media, *P. aeruginosa* becomes resistant to antimicrobial cationic peptides through the action of two distinct two-component regulatory systems, PmrA–PmrB and PhoP–PhoQ, which regulate LPS modifications (McPhee et al. 2006). Peptide-induced regulation of the LPS modification operon can also occur independently of these regulatory systems (McPhee et al. 2003; Moskowitz et al. 2004). This effect involves the *psrA* gene, which is upregulated in the presence of cationic antimicrobials and also mediates biofilm formation and swarming motility (Gooderham et al. 2008). All three systems are thought to mediate resistance by influencing the ability of the cationic agents to pass through the cell membrane (McPhee et al. 2003).

The selective permeability of the outer membrane to non-cationic agents is governed mainly by the properties of porins, which are integral outer membrane proteins that enclose a water-filled pore in the outer membrane of *P. aeruginosa* (reviewed in Hancock and Brinkman 2002). The major porin-forming protein is OprF, and the large exclusion limit of the *P. aeruginosa* outer membrane is thought to be mostly due to OprF because it is an inefficient route for taking up antibiotics (Hancock and Worobec 1998). In low-Mg^{2+} growth conditions, the PhoP–PhoQ system controls the overexpression of OprH, so that it (not OprF) becomes the major porin-forming protein (Macfarlane et al. 1999). OprH may occupy cation binding sites in the outer membrane, thus replacing the Mg^{2+} and contributing to membrane stability and antibiotic resistance. Porin OprD mediates the passage of carbapenem β-lactams, such as imipenem and meropenem. The major resistance mechanism to these antibiotics appeared to be mediated by the loss of the OprD porin, which was observed in as many as 50% of *P. aeruginosa* isolated from patients treated for longer than 1 week with imipenem (Quinn et al. 1988).

Multidrug Efflux Pumps

Four multidrug efflux pumps designated as complexes of Mex and Opr proteins have been characterized in *P. aeruginosa* and are named MexAB–OprM, MexCD–OprJ, MexEF–OprN, and MexXY–OprM. Each efflux system consists of an inner membrane drug-protein antiporter, an outer membrane porin (Opr), and a periplasmic membrane fusion protein (Poole 2001); collectively, the three components form a channel that allows substrates to be pumped directly from the bacterial cytoplasm to the extracellular environment. These pumps contribute to intrinsic drug resistance because their efflux activity can easily outpace the slower influx of inhibitors across the outer membrane, thus preventing the intracellular accumulation necessary for antibiotics to exert their lethal activity. Each of the four *mex* operons has its own regulatory gene that encodes a repressor; mutations in the repressor gene cause overproduction of their respective pumps, which results in increased drug resistance.

MexAB–OprM is the best characterized pump system, and most of the knowledge about efflux pump assembly comes from this system (Nehme and Poole 2007). It has the broadest substrate specificity known among multidrug transporters and is associated with efflux of β-lactams, chloramphenicol, tetracycline, quinolones, trimethoprim, novobiocin, and organic solvents (reviewed in Poole and Srikumar 2001). MexAB–OprM is the only pump that is constitutively expressed in wild-type strains, and transcription of *mexAB–oprM* is controlled by MexR, a repressor protein. MexXY–OprM contributes to the natural resistance of *P. aeruginosa* to aminoglycosides, erythromycin, and tetracycline; expression of MexXY is induced by exposure to these antimicrobial agents. A mutation in *mexR* leads to overexpression of the MexAB–OprM system, resulting in clinically relevant levels of resistance. All of the efflux systems extrude a wide variety of antimicrobial agent groups, but only MexXY–OprM was found to pump aminoglycosides and only MexAB–OprM demonstrated pumping of carbenicillin, sulbenicillin, ceftazidime, moxalactam, and aztreonam (Masuda et al. 2000).

The prevalence of efflux pump overproduction in clinical strains of *P. aeruginosa* may range from 14% to 75%. In a study by Beinlich et al. (2001), 12 multidrug resistant *P. aeruginosa* isolates from various animal infection sites all expressed significant levels of the MexAB–OprM efflux system, and two of them simultaneously expressed either MexAB–OprM and MexEF–OprN or MexAB–OprM and MexXY–OprN. Similarly, all 18 clinical isolates from dairy cows with *Pseudomonas* mastitis were found to express MexXY–OprM (Chuanchuen et al. 2008).

Acquired Resistance

Pseudomonas aeruginosa that produce metallo-β-lactamases, which mediate broad-spectrum β-lactam resistance, are increasingly implicated in nosocomial infections. The metallo-β-lactamase genes are located on integrons located on transposons, so the cassette can be easily transmitted (reviewed by Walsh et al. 2005). Acquisition of a multi-resistant phenotype through loss of porins or overexpression of efflux pumps has previously been described. The interaction of all of these resistance mechanisms leads to the highly resistant phenotype of *P. aeruginosa*. As a sample case, a single isolate was found to be resistant to anti-pseudomonal antimicrobials, including amikacin, ciprofloxacin, cefepime, ceftazidime, gentamicin, kanamycin, piperacillin/tazobactam, and tobramycin, and highly resistant (minimal inhibitory concentration [MIC] > 129 μg/ml) to imipenem and meropenem (Maniati et al. 2007). The isolate was analyzed and found to be deficient in porin OprD, to be overexpressing efflux pumps MexAB–OprM and MexXY–OprM, and to be harboring metallo-β-lactamase genes; thus, the high degree of carbapenem resistance was likely due to the interplay of these three resistance types (Maniati et al. 2007).

Resistance to Common Antibiotics

Recent data on antibiotic resistance in canine infections show that resistance to the quinolones and fluoroquinolones is quite common, but resistance to the carbapenems is extremely rare (Rubin et al. 2008). Less than 7% of the isolates are resistant to the β-lactams ceftazidime/clavulanic acid, ceftazidime, piperacillin/tazobactam, and cefepime; less than 8% of the isolates are resistant to the aminoglycosides gentamicin and amikacin (Rubin et al. 2008). A similar study reporting on canine pathological specimens found that imipenem, the quinolone antibiotics, marbofloxacin, and ciprofloxacin were the most effective (Seol et al. 2002). In humans, the International Nosocomial Infection Control Consortium studied resistance in *P. aeruginosa* and found that 52.4% of isolates are resistant to ciprofloxacin/

ofloxacin, 36.6% resistant to imipenem, 51.7% resistant to ceftazidime, and 50.8% resistant to piperacillin (Rosenthal et al. 2008). Several studies have suggested that the activity of a β-lactam may be enhanced by the addition of an aminogoycoside, but other studies have concluded that there is no difference in human mortality when combination therapy is compared with monotherapy with β-lactams.

IMPACT OF WHOLE GENOME SEQUENCES

Currently, several species of *Pseudomonas* have been targeted for genome sequencing. Multiple strains of *P. aeruginosa*, *P. putida*, *P. fluorescens*, and *P. syringae* have been sequenced in addition to single strains of *Pseudomonas mendocina*, *Pseudomonas entomophila*, and *Pseudomonas stutzeri*. These sequences and the annotation data are stored in the *Pseudomonas* Genome Project website (Winsor et al. 2005; v2.pseudomonas.com). Recently, sequencing efforts have been revolutionized by the massively parallel picolite reactor platform, a pyrosequencing technique developed by 454 Life Sciences Corporation (Branford, CT) that has the capability of reading millions of nucleotides per hour. A challenge is the bioinformatics effort to fully annotate these genomes, which lags behind the sequence data. For example, the reference strain PAO1 was sequenced in the year 2000, but to date, assignment of function has been established for only about 20% of the roughly 6000 open reading frames.

Comparison of the completed *Pseudomonas* genomes can provide a glimpse into the evolution of *Pseudomonas* and allow a greater understanding of the organism. Highly variable segments of genomes can be identified by comparison to the others, and specific genomic islands associated with cell physiology can be discovered. Recent work suggests that all *P. aeruginosa* strains possess a common “core genome,” which is characterized by the conserved order of the genes, a low average nucleotide divergence, and the presence of multiple alleles subject to diversifying selection at a few variable loci, such as the O antigen or pyoverdine locus. In addition to this core genome is the “accessory genome” consisting of a variable set of genomic islands that are not necessarily found in most *P. aeruginosa* strains. This gives a useful working description of the *P. aeruginosa* genome as a mosaic of conserved core and variable accessory segments (Tümmler 2006).

A recent work used a high-throughput microarray-based genotyping method to survey genomes of several *P. aeruginosa* strains using single-nucleotide polymorphisms to represent the core genome and a set of genomic markers that identify genomic islets to detect the accessory genome (Wiehlmann et al. 2007b). The results of the analysis were interesting as it noted that major clones were versatile in their habitat and geographic origin; for example, the sequenced reference strain PAO1 was found transcontinentally, and another strain, the PA14 clone, was found in both patients and aquatic habitats (Wiehlmann et al. 2007b). Another important finding was that the core and accessory genome were not randomly assembled, but rather individual clones prefer a specific repertoire of accessory segments, indicating that only certain combinations are tolerated at specific loci. The mechanisms and implications of this are not yet fully understood.

The availability of genome sequences has also impacted research into potential novel virulence determinants. One system to screen for new virulence factors is signature-tagged mutagenesis (STM). In this technique, predetermined “tagged” sequences are designed as components of transposons that are then used to induce mutation. Insertion of these tagged transposons causes random mutations. Mutants with reduced virulence are identified based on subsequent infection studies. STM studies can be carried out in a variety of hosts or selection systems, which allows the importance of virulence factors in different habitats or hosts to be tested. In some cases, a remarkable degree of conservation has been found in the virulence mechanisms used by *P. aeruginosa* to infect hosts of divergent evolutionary origins, such as animals and plants (Rahme et al. 2000), but a recent STM study found that certain mutants were attenuated in a worm infection model but displayed wild-type virulence when instilled into the airways of mice (Wiehlmann et al. 2007a). Thus, the accessory genome could be thought to contain specific determinants essential for virulence in one species but not necessarily of importance in a phylogenetically distant host.

Several other techniques take advantage of the genome sequence data, such as the *in vivo* expression technology (IVET) system that positively selects for bacterial genes that are specifically induced during host infection. Using the IVET system to examine virulence genes of *P. aeruginosa* in a neutropenic mouse infection model, Wang et al.

(1996) identified 22 genetic loci that were specifically induced *in vivo*. Although one of the genes encodes a well-studied virulence factor, a pyochelin receptor that is involved in iron acquisition, the other 21 genes encode novel virulence factors.

VACCINES

Vaccination against *P. aeruginosa* is problematic in several respects. The time of administration can be an issue since many *P. aeruginosa* infections are incidental wound infections that cannot be predicted beforehand. Still, vaccines are of interest for at-risk populations, such as mink and chinchillas, and the human CF community (patients with this genetic disorder are predisposed to chronic infections by *P. aeruginosa*). However, if an infected individual is given the vaccine, worsening of clinical symptoms and serum sickness may result. Development of useful vaccines has been challenging, and no human vaccine has yet been successful for prophylaxis toward the spectrum of *P. aeruginosa* infections.

The only animal vaccine of practical relevance against *P. aeruginosa* infections is for use in mink and chinchillas. This multicomponent vaccine consists of the so-called original endotoxic protein (OEP), which is a protein antigen normally complexed with LPS in the cell wall, and toxoids of alkaline protease and elastase. This vaccine was effective in preventing an epidemic in mink of hemorrhagic pneumonia due to *P. aeruginosa* (Aoi et al. 1979; Homma et al. 1983). Repeated vaccination has been identified as a possible cause of glomerular disease in mink, but no link to use of the *Pseudomonas* vaccine itself was indicated (Newman et al. 2002). There are no other *Pseudomonas* vaccines available for use in humans or animals, but numerous attempts have been made.

Vaccine development against *P. aeruginosa* has been recently reviewed by Döring and Pier (2008). Briefly, antigens such as LPS, surface polysaccharides, polysaccharide-protein conjugates, flagella, outer membrane proteins, pili, whole formalin-killed cells, live-attenuated *P. aeruginosa* and *Salmonella enterica* strains expressing *P. aeruginosa* antigens, and DNA sequences have been tested. Of these, only polysaccharide-based or flagella-based vaccines and passive immunization techniques have progressed to phase II and III studies.

The first approaches to vaccine development focused mainly on the use of LPS as the antigen. This was an attractive approach because LPS is a predominant cell surface antigen and a major component of the cell wall. It could theoretically be used to generate opsonizing antibodies against infecting strains. Unfortunately, LPS-based vaccines for *P. aeruginosa* have never gained clinical acceptance due to the endotoxic nature of the vaccine and the poor immunogenicity of purified polysaccharide. The best known anti-*Pseudomonas* vaccines are Pseudogen®, PEV-01, and Aerugen®. Both Pseudogen and PEV-01 have provided significant protection against experimental acute pneumonia. However, when Pseudogen was tested in CF patients already infected with *P. aeruginosa*, the organism was not cleared from the airways, and the patients did worse clinically than the unvaccinated control group (Pennington et al. 1975). In tests in other patient groups, the high adverse reaction rate was a major drawback, and development of Pseudogen has been terminated. Development of PEV-01 has also been stopped after a study in CF patients in which the vaccinated group had worse overall clinical status than the control group despite detection of specific antibody production in those that received the vaccine (Langford and Hiller 1984). Aerugen, an octavalent conjugate vaccine containing O polysaccharides from several serotypes of *P. aeruginosa* covalently coupled to ExoA, conferred protection in animals to the serotypes contained in the vaccine (Cryz et al. 1989). In a study of 26 CF patients not infected with *P. aeruginosa*, vaccination with Aerugen resulted in significantly fewer vaccinated patients that were colonized by the end of the 10-year observation period than the control group (Lang et al. 2004). Another study of Aerugen in CF patients was stopped because no difference between treatment groups could be discerned; the future for this vaccine seems uncertain (Döring and Pier 2008).

Controlled clinical trials of passive immunization were attempted using healthy donors immunized with either PEV-01 or Aerugen. The results from the PEV-01 vaccine were promising, but no recent work has followed up on the study, and PEV-01 is no longer being developed. Patients receiving hyperimmune globulin from the Aerugen-vaccinated donors had more adverse reactions. Currently there is no passive immunotherapy scheme that has been deemed safe enough to be licensed.

An O antigen-specific vaccine was tested in sheep and was shown to prevent dissemination but not

lung injury when given prior to inoculation with *P. aeruginosa* (Pittet et al. 1995). Administration of antibodies to the respiratory tract was able to prevent both dissemination and lung injury, possibly because the circulating antibodies need lung damage to enter the lungs, whereas the administered antibodies were already present at the site of infection (Pittet et al. 1995; Scarff and Goldberg 2008). An alternate method of LPS-based vaccination has been studied, in which a live, attenuated vaccine strain of *Salmonella* was engineered to express the LPS O antigen of *P. aeruginosa* serotype O11 (DiGiandomenico et al. 2004). Intranasal immunization with the recombinant vaccine strain conferred protection against infection by multiple serogroup O11 strains in the lung, cornea, and burn-wound infection models. Passive transfer of vaccine antisera did not provide protection against infection with a serotype O6 strain, indicating that protection correlated with the presence of anti-O11 antibodies (DiGiandomenico et al. 2007). This demonstrates how the serological diversity of *P. aeruginosa* strains further complicates vaccine development. The same recombinant *Salmonella* vaccine strain was used to vaccinate immunocompromised mice, which are hypersusceptible to *P. aeruginosa* infection. Vaccination decreased the susceptibility of the mice and slowed down the progression of disease, and antibodies were also protective when given at the time of infection (Scarff and Goldberg 2008). Since there are 20 serotypes of *P. aeruginosa*, the group hopes to create a multivalent vaccine strain that will be protective against the most prevalent serotypes, but this will be challenging.

A flagella-based vaccine was recently shown to be protective in human CF patients (Döring et al. 2007). Although the number of fully vaccinated patients who later became colonized with *P. aeruginosa* was lower than the control group, vaccination was not shown to prevent chronic infection due to a lower-than-expected rate of infection in the placebo group (Döring and Pier 2008). Manufacture of this vaccine has also been terminated. Other efforts have attempted active and passive immunization using the *P. aeruginosa* PcrV protein, essential for the TTSS, and this enhanced survival in an acute lung infection model (Sawa et al. 1999). Antibodies against PcrV have been protective in animal models of *P. aeruginosa* sepsis. Many other targets are being exploited for potential vaccines but have not yet progressed to phase II/III clinical trails. An issue for testing vaccines in a patient group at risk for *P. aeruginosa* infection is the improved antibiotic therapy regimens that impairs proper evaluation of the vaccine efficacy (Döring and Pier 2008).

CONCLUSIONS AND FUTURE PROSPECTS

Due to its remarkable physiological versatility, *P. aeruginosa* is ubiquitous. Clonal strains are found in both environmental and clinical isolates, underscoring the opportunistic nature of *P. aeruginosa* infections. Pathogenesis of this organism is complex because it possesses a vast array of virulence factors, and the bacterium can cause a broad range of diseases in compromised hosts. Evidence has been presented for both a common set of virulence mechanisms that are active in a range of phylogenetically diverse hosts, as well as specific virulence determinants present in the accessory genome that mediate interaction with only certain hosts. Without a useful vaccine, treatment of *Pseudomonas* infections requires appropriate antibiotic treatment, but this is complicated by the innate resistance and ever-increasing acquired multidrug resistance properties of *P. aeruginosa*. Current research is therefore directed toward identifying and characterizing novel virulence determinants required by the organism to establish or maintain an infection. These studies may generate novel antibiotic targets or suggest more unusual therapeutic agents to be used in conjunction with traditional antibiotic therapy.

For example, bacteria living in biofilms resist conventional antimicrobial treatments. The mechanism of antimicrobial resistance associated with biofilm grown cells has not been clearly defined, but it has been suggested that it could be multifactorial. One of the strategies proposed by researchers to deal with biofilm-associated drug resistance is to develop inhibitors of the regulatory quorum-sensing network in order to abrogate the bacteria's ability to form biofilms or to activate key virulence factors. *N*-decanoyl cyclopentylamide inhibited production of elastase, pyocyanin, and rhamnolipid and biofilm formation by interfering with the *las* and *rhl* systems (Ishida et al. 2007). A collection of screening systems, called quorum-sensing inhibitor selectors, have been constructed to enable the identification of new quorum-sensing inhibitors, such as garlic extract and 4-nitro-pyridine-*N*-oxide (Rasmussen

et al. 2005). Application of these two quorum-sensing inhibitors significantly reduced *P. aeruginosa* biofilm tolerance to tobramycin, as well as virulence in a *Caenorhabditis elegans* pathogenesis model (Rasmussen et al. 2005). Further use of the quorum-sensing inhibitor selector showed that azithromycin, ceftazidime, and ciprofloxacin decrease the expression of a range of quorum-sensing-regulated virulence factors, possibly mediated by changes in membrane permeability (Skindersoe et al. 2008). Thus, the interplay between quorum sensing and antibiotic treatment is of current interest.

A novel method of combined phenotypic and chemical genetics high-throughput screen in baker's yeast has been designed to study bacterial toxins and to identify their inhibitors (Arnoldo et al. 2008). Data from this screen helped to identify exosin, the first known inhibitor of *P. aeruginosa* ExoS (Arnoldo et al. 2008). In the future, exosin and other novel inhibitors can be tested for protective ability against *P. aeruginosa* infection in humans or animals. Inhibition of c-di-GMP production is another possible target for new antimicrobial agents effective against biofilm-related diseases since c-di-GMP binding is necessary for the activation of several polysaccharides required for biofilm formation (Lee et al. 2007). Perhaps multifaceted approaches to *Pseudomonas* infection treatment, combining antibiotics as well as quorum sensing and virulence factor inhibitors, will prove to be effective in the future. Investigation of the pathogenic mechanisms and antibiotic resistance of the *Pseudomnas* species will remain a rich area of research until better tools for the management of multidrug resistant *Pseudomonas* infections have gained common clinical acceptance.

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24
Moraxella

J. A. Angelos

INTRODUCTION

The genus *Moraxella* is the type genus of the family *Moraxellaceae* in the class Proteobacteria (Rossau et al. 1991). Moraxellae are gram negative bacteria that are short rods, coccobacilli, or cocci; 20 species have been described. Eleven of these were isolated from healthy animals or animals with disease (table 24.1). Of the five *Moraxella* species isolated from animals exhibiting disease (table 24.1), our most complete understanding of pathogenesis exists for *Moraxella bovis*.

A relatively recently recognized species, *Moraxella bovoculi* was isolated from IBK-affected dairy and beef calves in northern California, United States, in 2002 (Angelos et al. 2007c). It is likely that *M. bovoculi* has existed in the cattle population for a long time and that gram-negative cocci previously isolated from cattle with IBK were, in fact, *M. bovoculi* that were designated as "*M. ovis*," "*M. ovis-like*," "*Branhamella ovis*," or "*Branhamella ovis-like*" species. *Branhamella* was previously used to refer to the coccoid *Moraxellae*. Molecular tools now make it possible to more easily distinguish *M. ovis* and *M. bovoculi* (Angelos and Ball 2007a). The primary focus of this chapter will be those pathogenic features that are described for *M. bovis*, the etiologic agent of IBK. Where knowledge of pathogenic features of *M. bovoculi* and *M. ovis* exists, these will also be discussed.

INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

Infectious bovine keratoconjunctivitis (IBK) is the most common eye disease of cattle. All breeds of

Table 24.1. Species of *Moraxella* Isolated from Animals

Species of *Moraxella*	Animal source	Reference
Moraxella bovis	Cattle with infectious bovine keratoconjunctivitis (IBK, "pinkeye")	Allen 1919
Moraxella bovoculi	Cattle with IBK	Angelos et al. 2007c
Moraxella ovis	Sheep with conjunctivitis	Lindqvist 1960
Moraxella oblonga	Oral cavity of sheep	Xie and Yokota 2005
Moraxella canis	Muzzles of healthy dogs and cats	Jannes et al. 1993
Moraxella boevrei	Nasal passage of healthy goats	Kodjo et al. 1997
Moraxella caprae	Nasal passage of healthy goats	Kodjo et al. 1995
Moraxella caviae	Pharynx of healthy guinea pigs	Pelczar et al. 1949
Moraxella cuniculi	Nasopharynx of rabbits	Berger 1962
Moraxella equi	Horse with conjunctivitis	Hughes and Pugh 1970
Moraxella anatipestifer	Septicemic ducklings	Hendrickson and Hilbert 1932

cattle are susceptible to infection, although lower incidences of IBK have been reported in Brahman cattle and in cattle with increased pigmentation at the ocular margins (Frisch 1975; Ward and Neilson 1979). Cattle with IBK exhibit corneal ulcers, corneal edema, photophobia, blepharospasm, and lacrimation. Corneal ulcerations associated with *M. bovis* in cattle heal with varying degrees of corneal scarring; severe scarring may result in reduced vision. In the most severe cases, corneal rupture occurs leading to permanent blindness.

Corneal ulceration associated with IBK has an economic consequence; such losses are associated with reductions in animal weight gain that depends on the severity of the infection/scarring, and on whether one or two eyes are affected. Along with the economic effects of infection and treatment, the negative impact on individual animal welfare associated with corneal ulceration cannot be ignored, and efforts to understand and prevent IBK are necessary and important for cattle producers and veterinarians.

Early reports on the etiology of IBK suggested associations with infectious bovine rhinotracheitis (IBR) virus (Sykes et al. 1962; Mohanty and Lillie 1970), and mycoplasma (Langford and Dorward 1969). While it is now known that IBK is not caused by these agents, infection with these organisms remains important insofar as they may promote ocular injury (Pugh et al. 1970, 1976; George et al. 1988) as well as increase ocular and nasal secretions that may facilitate the transmission of *M. bovis* between animals are concerned.

Other risk factors for IBK include flies, ultraviolet radiation, and direct mechanical trauma from seed awns such as foxtails. *M. bovis* can survive for 3 days on the outside surface (Steve and Lilly 1965) and 2 days in the alimentary tract of face flies (*Musca autumnalis*; Glass et al. 1982). Development of IBK in cattle exposed to face flies that were exposed to *M. bovis* culture media has been reported (Arends et al. 1984). Fly control using insecticide impregnated ear tags or back/face rubbers can be an effective means of reducing IBK in cattle populations (Gerhardt et al. 1982). An association between IBK and ultraviolet (UV) radiation exposure is documented (Hughes et al. 1965, 1968; Lepper and Barton 1987). The corneas of UV-exposed calves develop corneal epithelial cell degeneration (Vogelweid et al. 1986) that is believed to predispose to *M. bovis* infection and IBK. Seed awns can mechanically scratch the corneal surface and predispose the eye to an *M. bovis* infection and development of IBK; some producers will clip mature grasses prior to turning cattle out to graze in order to minimize the risk of plant awns contributing to IBK.

Treatment of IBK is centered around either systemic or local (in the eyelids or bulbar conjunctiva) administration of antibiotics. As *M. bovis* is susceptible to many antimicrobials, producers and veterinarians have a wide variety of therapeutic options.

Many commercial vaccines are available to prevent IBK; autogenous bacterins produced by vaccine manufacturers from isolates supplied by veterinarians are also used. The efficacy of pinkeye vaccines can be altered by management factors as they relate to timing of vaccination; it is recommended that a vaccine protocol be initiated approximately 6–8 weeks before IBK cases are first anticipated in a herd to allow adequate time for the development of a protective antibody response.

PATHOGENESIS OF *MORAXELLA BOVIS*

Introduction

Experimental *M. bovis* infection of cattle results in corneal ulcers, conjunctival erosions, and the accumulation of fibrin, neutrophils, and bacteria within the corneal stroma (Rogers et al. 1987a, 1987b). Most published work on *M. bovis* pathogenesis has focused on those proteins that allow the organism to: (1) attach to the cornea; and (2) cause damage to the corneal epithelium. The *M. bovis* attachment proteins that are best characterized are pili. The subsequent damage to the corneal epithelium following attachment is attributed to a repeats in the structural toxin (RTX) toxin (cytotoxin; also called cytolysin or hemolysin).

Pili and cytotoxin have clearly demonstrated roles in pathogenesis. However, *M. bovis* produces a variety of enzymes that, while not directly proven to be involved in pathogenesis and not studied in as much detail as *M. bovis* pili or cytotoxin, may be involved in the development of IBK. These include a variety of hydrolytic enzymes including C4 esterase, C8 esterase-lipase, C14 lipase, phosphoamidase, phosphatase, leucine and valine amino-

peptidases and gelatinase (Frank and Gerber 1981), fibrinolysins (Nakazawa and Nemoto 1979a), proteins with hemolytic activity (Nakazawa and Nemoto 1979b), and cell detachment proteins (Marrion and Riley 2000). *M. bovis* proteins besides pili may be involved in corneal attachment; these proteins include filamentous hemagglutinin (Kakuda et al. 2006). As discussed below, a phospholipase and proteins that are involved in iron acquisition have also been identified in *M. bovis* and may be important in pathogenesis. The discussion below will first describe the more recently characterized proteins that may be involved in *M. bovis* pathogenesis, followed by discussions of *M. bovis* pili, cytotoxin, humoral immune responses following *M. bovis* infections, and vaccination to prevent IBK.

Established or Putative Virulence Factors

Phospholipase B

In 2001, an approximately 66-kDa secreted protein that has phospholipase B activity was described in *M. bovis* (Farn et al. 2001). It is now known that this protein, designated PLB and encoded by *plb*, belongs to the GDSL (Gly-Asp-Ser-Leu) family of lipolytic proteins (Shiell et al. 2007). While not yet proven to be a key feature of *M. bovis* pathogenesis, it is likely that *M. bovis* PLB is involved in pathogenesis and may be a suitable candidate antigen for inclusion in IBK subunit vaccines.

Iron Acquisition and Protein Expression

Early work on iron-acquisition mechanisms by *M. bovis* demonstrated the presence of transferrin-binding proteins 1 and 2 (Tbp1 and Tbp2) in membrane preparations; these binding proteins were specific for bovine but not ovine or caprine transferrin (Yu and Schryvers 1994). Subsequent work identified transferrin-binding protein encoding genes A and B (*tbpA* and *tbpB*) as well as lactoferrin-binding protein (LBP) encoding genes A and B (*lbpA* and *lbpB*) in *M. bovis* (Yu and Schryvers 2002). Isogenic mutants deficient in lactoferrin-binding protein A and/or B were unable to utilize bovine lactoferrin (bLf) for growth and demonstrated reduced bLf binding. Based on other work with *Moraxella catarrhalis* in which bactericidal activity was shown with rabbit antisera against recombinant lactoferrin-binding protein B (Du et al. 1998), it was postulated that *M. bovis* LBPs may be suitable candidate antigens for incorporation in vaccines against IBK.

Initial investigations into the effect of iron availability on *M. bovis* protein expression led to the discovery that two outer membrane proteins (OMPs) and putative siderophores could be induced under iron-limiting conditions (Fenwick et al. 1996). These investigators demonstrated that *M. bovis* expressed iron-binding ligands that were most likely siderophores when grown in a low-iron environment. One of the OMPs was an ~78-kDa protein and was postulated to be a siderophore receptor. The other OMP identified by Fenwick et al. was ~104 kDa, close to the ~99 kDa size of an *M. bovis* cytotoxin (MbxA; Angelos et al. 2001). The expression of RTX-like proteins under iron-limiting growth conditions was previously demonstrated for *Neisseria meningitidis* (Thompson et al. 1993).

Given that protein expression is induced in *M. bovis* in response to low environmental iron concentrations, there must be mechanisms for regulation of this response. One such mechanism was elucidated in 2003 when a homolog of the ferric uptake regulator (*fur*) gene of *M. bovis* was identified, cloned, and characterized (Kakuda et al. 2003a). The expression of Fur decreased when iron was available; Fur was also shown to bind to DNA fragments upstream of the *fur* gene. An ~79-kDa iron repressible protein designated IrpA is negatively regulated by Fur; IrpA deficient strains of *M. bovis* have reduced ability to grow under conditions where the iron source in the medium is bovine transferrin or bovine lactoferrin (Kakuda et al. 2003b).

Filamentous Hemagglutinin

Two large open reading frames (ORFs) designated *flpA* and *flpB* were recently reported on a 44-kb plasmid, pMBO-1, of *M. bovis* strain Epp63 that encodes proteins that have homology with the *Bordetella pertussis* filamentous haemagglutinin (FHA), which allows *B. pertussis* to adhere to mucosal surfaces (Kakuda et al. 2006). An additional ORF possibly involved in the secretion of putative *M. bovis* filamentous haemagglutinins was also reported on the same plasmid. Both FlpA and FlpB have integrin-binding sequences (Ruoslahti 1996) including LDV (leu-asp-val) and IDS (ile-asp-ser; involved in $\alpha_4\beta_1$ integrin binding), RLD (arg-leu-asp; $\alpha_V\beta_3$ and $\alpha_M\beta_2$ integrin binding), and IET (ile-glu-thr; $\alpha_L\beta_2$ binding) sequences (Kakuda et al. 2006). Along with these similarities, FlpA and

FlpB share similarity with the *B. pertussis* FHA precursor FhaB in that both have a putative ~77 amino acid-long signal sequence possibly involved in Sec-dependent transport.

Kakuda et al (2006) also reported another ORF on pMBO-1 encoding a putative Flp accessory protein (Fap) that has homology with the accessory protein FhaC that is necessary for interaction with *B. pertussis* FHA as it traverses the periplasmic space on its way to the cell surface. Reverse transcription-polymerase chain reaction (RT-PCR) analysis demonstrated the presence of *flpA*, *flpB*, and *fap* gene transcripts in RNA extracted from *M. bovis* Epp63 grown *in vitro* (Kakuda et al. 2006). The *flp* and *fap* genes were identified in all but one isolate in a collection of geographically diverse isolates of *M. bovis*. Further experiments designed to evaluate infectivity of *flp* mutant strains of *M. bovis* will be needed to fully evaluate the role of filamentous hemagglutinin in *M. bovis* pathogenesis.

Important roles for proteins other than pili (see below) in *M. bovis* pathogenesis are likely. For example, it has been shown that non-piliated strains of *M. bovis* can adhere, albeit less efficiently than piliated strains, to various cell types, suggesting a role for adhesins besides pili in the attachment of *M. bovis* to cell surfaces (Annuar and Wilcox 1985).

Pili

A necessary feature of *M. bovis* pathogenesis is the expression of pili, cell surface projections that are composed of individual repeating subunits of pilin. During passage in the laboratory, piliated *M. bovis* often stop expressing pili, resulting in a smooth phenotype. The pili of *M. bovis* are peritrichously distributed 6.5-8.5 nm diameter elongated unbranched filaments on the surface of rough but not smooth colony types of *M. bovis* (Simpson et al. 1976). These pili are N-methyl phenylalanine type (type 4; Patel et al. 1991; Heinrich and Glasgow 1997). Piliated strains of *M. bovis* adhered better than non-piliated strains to various bovine cell surfaces and detachment of pili from *M. bovis* by use of magnesium chloride treatment greatly reduced the ability of *M. bovis* to adhere to these surfaces (Annuar and Wilcox 1985). The importance of pili in *M. bovis* pathogenesis was subsequently demonstrated *in vivo*: piliated *M. bovis* strain Epp63 induced IBK while non-piliated strains did not, and vaccines derived from piliated cultures of *M. bovis* protected against IBK in an experimental challenge (Jayappa and Lehr 1986).

In 1986, six serogroups of *M. bovis* pili, designated I through VI, were described based on ELISA methods (Lepper and Hermans 1986). Following this work, a larger study with more *M. bovis* strains and using slide agglutination, ELISA, and tandem-crossed immunoelectrophoresis reported seven pilus serogroups designated A through G (Moore and Lepper 1991).

It is likely that some of the observed differences in *M. bovis* vaccine efficacy is related to pilin serogroup differences between herd strains and vaccinal strains. In addition to serogroup differences, an added layer of diversity between strains exists because of two distinctly different phases of pilin that *M. bovis* strain Epp63 can express: I (previously the α form) and Q (previously the β form) pili; these two distinct forms result from the inversion of a 2-kb region of DNA (Marrs et al. 1988). When I and Q pili-expressing variants of *M. bovis* strain Epp63 were examined to determine differences in infectivity, Q pili strains were found to be more infectious than I pili strains (Ruehl et al. 1993a, 1993b). *In vivo* switching between the two pilus forms has been observed and led to the conclusion that Q pili were important for colonization of the bovine cornea, while I pili were involved in maintaining an established infection (Ruehl et al. 1993b). Such switching might be important in *M. bovis* evasion of host immune responses.

If pilus type switching occurs in other serogroups of *M. bovis*, the antigenic diversity of *M. bovis* pilus types is expected to be high. Cross-protection might, however, be possible between pilus-based vaccines that incorporate antigens comprised of the amino end of pilin that is highly conserved among serogroups (Atwell et al. 1994). Cyanogen bromide cleavage of pilin protein preparations exposed shared conserved antigenic determinants between heterologous *M. bovis* strains (Greene et al. 2001a, 2001b); such treatment could be employed in developing pilus-based vaccines that cross-protect among various *M. bovis* serogroups. An experimental recombinant subunit vaccine that incorporated the conserved amino terminus of one *M. bovis* strain coupled with the carboxy terminus of the *M. bovis* cytotoxin has been tested in the field against naturally occurring IBK (Angelos et al. 2007b); however, it is not known whether this vaccine antigen imparts

an advantage against heterologous challenge with different *M. bovis* pilin serogroups.

Recently, methods for assaying and improving piliation of *M. bovis* grown in large bioreactors for vaccine production have been described (Prieto et al. 2003, 2008). In addition to the improvement of piliation by use of bubble column bioreactors versus stirred and sparged bioreactors to reduce mechanical shear forces on bacterial cells, it was found that the addition of carboxymethylcellulose to culture medium improved levels of piliation without altering pili antigenic properties (Prieto et al. 2008).

Cytotoxin (Hemolysin/Cytolysin)

Along with pili that are necessary for attachment, the pathogenesis of *M. bovis* also depends on the expression of a cytotoxin (also called hemolysin or cytolysin) that has calcium-dependent hemolytic, corneotoxic, and leukotoxic properties (Kagonyera et al. 1989a, 1989b; Hoien-Dalen et al. 1990; Beard and Moore 1994; Gray et al. 1995). Strains of *M. bovis* that are nonhemolytic are not pathogenic in cattle (Pugh and Hughes 1968; Beard and Moore 1994). The importance of *M. bovis* hemolysin/cytotoxin as a critical virulence determinant was demonstrated by intra-corneal injection of a hemolytic fraction from pathogenic *M. bovis* (Beard and Moore 1994). Corneal lesions that were similar to lesions produced in naturally occurring IBK were induced; equivalent fractions produced from nonhemolytic *M. bovis* did not cause ocular lesions. When grown on blood agar, hemolytic colonies of *M. bovis* are surrounded by a zone of β hemolysis. When erythrocytes are exposed to the *M. bovis* hemolysin (cytotoxin), potassium efflux, colloid-osmotic cell swelling, and lysis occur. Because these effects can be inhibited with osmotic protectants, it is believed that the hemolysin causes the formation of transmembrane pores in target cell membranes (Clinkenbeard and Thiessen 1991).

Cattle recovered from IBK develop an anti-hemolysin antibody response (Ostle and Rosenbusch 1985; Hoien-Dalen et al. 1990; Billson et al. 1994) that can neutralize the hemolysin from various strains of *M. bovis* (Ostle and Rosenbusch 1985), and calves that are vaccinated with *M. bovis* hemolysin are protected against heterologous *M. bovis* challenge (Billson et al. 1994). These studies have helped to establish that the *M. bovis* cytotoxin is a key feature of *M. bovis* pathogenesis as well as a suitable vaccine antigen to induce protection against IBK associated with diverse strains of *M. bovis*.

The possibility that the *M. bovis* cytotoxin might belong to the RTX family of bacterial pore-forming toxins was postulated because a monoclonal antibody against HlyA, an RTX toxin expressed by uropathogenic *Escherichia coli*, recognized a 110-kDa protein in extracts from hemolytic but not nonhemolytic strains of *M. bovis* (Gray et al. 1995). Subsequently, a monoclonal antibody that could neutralize hemolytic activity of *M bovis* was described; this antibody recognized a 94-kDa protein from hemolytic *M. bovis* strains representing each of the different pilus serogroups (Billson et al. 2000).

Angelos et al. (2001) identified a gene, *mbxA*, that encodes an ~98.8-kDa protein *M. bovis* cytotoxin, MbxA, that is related to RTX toxins of gram-negative bacteria. Other gram-negative bacteria of veterinary significance that encode RTX toxins include *E. coli* and many species of *Actinobacillus*, *Pasteurella*, and *Haemophilus*. In MbxA there are amino acid sequence motifs that are characteristic of RTX toxins (Angelos et al. 2001), including glycine-rich repeats in the carboxy terminus. Four of six such repeats in MbxA match the predicted consensus sequence (Leu/Val-Xaa-Gly-Gly-Xaa-Gly-Asn/Asp-Asp-Xaa [L/V-X-G-G-X-G-N/D-D-X)]) for glycine repeats in RTX toxins. In addition, key lysine residues that are likely targets for activation of the toxin by fatty acylation are present in MbxA. Both hemolytic and cytotoxic activities of a preparation of native *M. bovis* cytotoxin were neutralized by rabbit antiserum against the carboxy terminus of MbxA (Angelos et al. 2001).

Following the discovery of MbxA, sequencing of the DNA flanking *mbxA* revealed the presence of a classical RTX operon (Angelos et al. 2003). Classical RTX operons are composed of four genes arranged 5’-C-A-B-D-3’. The best characterized RTX operon is *hly* from uropathogenic *E. coli*. HlyC activates the structural RTX toxin proper (HlyA) by fatty acylation of conserved lysine residues (Issartel et al. 1991; Stanley et al. 1994). HlyB and HlyD are required for the extracellular transport of HlyA (Koronakis et al. 1992). A secretion accessory protein, TolC, is necessary for the transport of HlyA out of the cell; *tolC* is unlinked to the Hly operon in *E. coli* (Wandersman and Delepelaire 1990). In *M. bovis*, the *mbx* operon genes were designated *mbxC* (toxin activating protein); *mbxA* (structural

cytotoxin); *mbxB* (transport); and *mbxD* (transport) genes. In addition, a gene encoding a protein related to TolC flanks *mbxD* and is probably necessary for cytotoxin secretion (Angelos et al. 2003).

Complete classical RTX operons have been identified in *M. bovoculi* and *M. ovis*, along with a closely linked gene flanking the D gene that encodes a putative secretion accessory protein homologous to TolC (Angelos et al. 2007a). In *M. bovoculi*, the operon was designated the *mbv* operon and is comprised of *mbvCABDtolC* genes; in *M. ovis* the operon was designated the *mov* operon and is comprised of *movCABDtolC* genes. This finding confirmed a previous prediction that *M. ovis* expressed an RTX toxin (Cerny et al. 2006). Rabbit polyclonal antiserum to the carboxy terminus of *M. bovoculi* MbvA neutralized hemolytic activity of *M. bovoculi* and *M. ovis*, but did not neutralize hemolytic activity of *M. bovis*. Rabbit polyclonal antiserum to the carboxy terminus of *M. bovis* MbxA only partially neutralized hemolytic activity of *M. bovoculi* and *M. ovis* (Angelos et al. 2007a). *M. bovoculi* MbvA and *M. ovis* MovA have ~98% identity in deduced amino acid sequence; the deduced amino acid sequence of *M. bovis* MbxA is ~83% identical to those of MbvA and MovA (Angelos et al. 2007a).

For three other RTX toxins, *E. coli* α-hemolysin, *A. actinomycetemcomitans* leukotoxin, and *M. haemolytica* leukotoxin, the cellular receptor on leukocytes has been identified as a β_2 integrin (Lally et al. 1997; Ambagala et al. 1999; Li et al. 1999). While the white blood cell receptor to which *M. bovis* cytotoxin binds has not been reported, it is likely that it is a β_2 integrin.

Hemolytic strains of *M. bovis* are pathogenic and nonhemolytic strains are not considered pathogenic; however, the exact role of nonhemolytic *M. bovis* in nature and disease is not well understood. Nonhemolytic *M. bovis* have been isolated from asymptomatic cattle (Pugh and Hughes 1975), cattle exhibiting conjunctivitis (Kodjo et al. 1994), and simultaneously with hemolytic *M. bovis* in cattle with IBK (Pugh and Hughes 1975). Annual summer outbreaks of IBK are typical and could be due to cattle that harbor *M. bovis* asymptomatically (Pugh and Hughes 1975; Pugh and McDonald 1986). Shifting between hemolytic and nonhemolytic phenotypes has been documented (Pugh and Hughes 1968).

The *mbxCABD* genes were not detected in nonhemolytic strains of *M. bovis* (Angelos et al. 2003). Further examination of the DNA flanking the 5′ and 3′ ends of *mbxCABDtolC* in hemolytic *M. bovis* revealed that approximately 700 bp imperfect repeats flank the operon (Hess and Angelos 2006; fig. 24.1). Nonhemolytic strains of *M. bovis* that were examined had only one or no such repeats, as well as ORFs that were identical to those flanking the repeats from hemolytic *M. bovis*. The genetic organization, lower G + C content of *mbxCABDtolC*, and flanking repeats in hemolytic *M. bovis*, as well as the presence or absence of flanking repeats in nonhemolytic *M. bovis*, suggests that the *M. bovis mbx* operon is located on a mobile genetic element, and supports the concept that this region is a pathogenicity island (PAI). This is the first such element demonstrated in *M. bovis* strain Tifton I, and has been designated PAI $I_{\text{Tifton I}}$ (Hess and Angelos 2006). The possibility that nonhemolytic *M. bovis* could become hemolytic and potentially pathogenic by direct acquisition of the *mbx* operon is suggested by this work; whether or not this occurs *in vivo* has not yet been determined.

A method for partially purifying and stabilizing the *M. bovis* cytotoxin from culture supernatants has been described (George et al. 2004). This method employs diafiltration of culture supernatant and results in a cytotoxin-enriched product that is stable for up to 4 months at −80°C. The native cytotoxin prepared using this method was effective in stimulating protection against IBK (George et al. 2005).

Humoral Immune Response During *Moraxella bovis* Infections

In normal bovine lacrimal secretions, secretory IgA is the major immunoglobulin (Mach and Pahud 1971), and in experimentally induced IBK in calves, tear IgG1 and IgG2 concentrations increase (Pedersen 1973). In one early study, calves with more severe IBK were found to have higher lacrimal IgA titers to crude *M. bovis* antigen preparations than calves with less severe IBK (Nayar and Saunders 1975). A later study reported predominantly tear IgG responses to crude whole cell *M. bovis* antigens in calves with naturally occurring IBK, but concluded that specific *M. bovis* antibodies in lacrimal secretions did not prevent IBK (Killinger et al. 1978). Higher IgA than IgG titers were found in tears to nonspecific *M. bovis* antigens as detected by ELISA in calves infected with *M. bovis* (Bishop et al. 1982). In a small number of calves, both lacrimal (secretory IgA) and humoral (IgG) antibodies

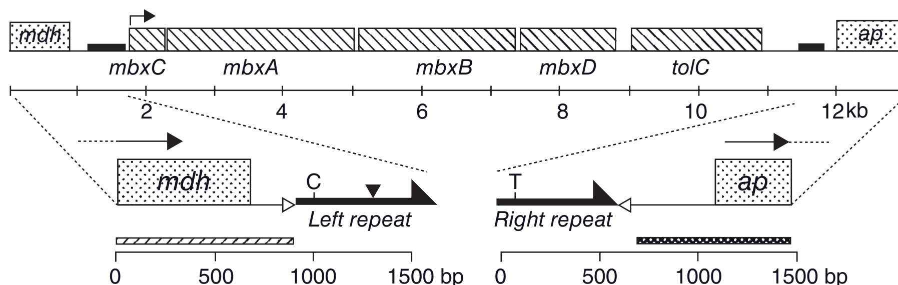

Figure 24.1. Schematic diagram depicting the arrangement of DNA elements flanking *mbxCABDtolC* within *M. bovis* Tifton I. The top panel shows approximately 13 kb of DNA, with the *mbxCABDtolC* genes flanked by ORFs tentatively identified as malate dehydrogenase (*mdh*) and aminopeptidase (*ap*). The bottom section of the figure represents an enlargement of the ORFs, and flanking left and right repeats, with scale bars (bp) indicated below the respective left and right hand regions; the hatched and checkerboard bars above the scale bars indicate sequences also found in common with nonhemolytic strains. The white triangle on the left side of the left repeat represents the sequence 5′-AAATCCT-3′, the inverse complement of which, 5′-AGGATTT-3′, is found on the right side of the right repeat, also indicated by a white triangle. Within the left and right repeats of *M. bovis* Tifton I, at position 27 is a sequence difference, C in the left repeat and T in the right repeat, indicated above them. The black triangle over the left repeat indicates an 80-bp insertion that is not present in the right repeat. With regard to this schematic, *M. bovis* T+ has an identical structure, with the exception of a matching T at position 27 of the left and right repeats (Hess and Angelos 2006; reprinted with permission from the Society for General Microbiology; copyright 2006).

against *M. bovis* whole cell antigen were reported to confer resistance to IBK as compared with a humoral IgG antibody response alone (Smith et al. 1989). Smith et al. (1989) concluded that serum antibodies directed against *M. bovis* might reduce the length and severity of clinical signs associated with IBK. In none of these studies were total antibody isotype responses measured in serum or ocular secretions. Furthermore, crude *M. bovis* antigen preparations were used in the aforementioned studies and so there is a lack of consistent information concerning the qualitative and quantitative aspects of the immune response that develops following *M. bovis* ocular infection and of the significance of this response in protection against IBK.

Vaccination to Prevent IBK

Early studies that reported reduced *M. bovis* infection rates and decreased occurrence of IBK after reexposure to *M. bovis* suggest that it may be possible to protect against IBK by vaccination (Hughes et al. 1968). Indeed, it has been shown that calves vaccinated intramuscularly with live *M. bovis* develop less IBK following challenge as compared with control calves (Hughes and Pugh 1971). Formalin-killed *M. bovis* has also been reported to be as effective as live cultures in preventing experimentally induced IBK (Hughes and Pugh 1972). Under field conditions, however, a formalin-killed autogenous bacterin was not protective (Hughes et al. 1976).

Efforts to identify suitable vaccine antigen candidates in *M. bovis* vaccines have led to studies of component vaccines. Calves that were vaccinated with pili could be protected from homologous challenge (Pugh et al. 1977). In another study, purified *M. bovis* ribosomes did not confer protection against experimentally induced IBK (Pugh et al. 1981). Bacterin-containing pili as well as corneal-degrading enzymes were protective in field trials, and the level of protection was correlated with the content of corneal-degrading enzyme in the vaccine (Gerber et al. 1988). The composition of the corneal-degrading enzymes in that study was not reported.

Despite the immunogenicity of pili, there is marked antigenic diversity between different pilus types due to the presence of pilin gene inversions (see above) and to differences between the amino acid composition of pili from different *M. bovis* serogroups. Limited antigenic cross-reactivity between heterologous pili (Ruehl et al. 1988; Lepper et al. 1995) and the emergence of novel pilus types

may lead to outbreaks of IBK (Vandergaast and Rosenbusch 1989). Such antigenic variability would be expected to reduce the overall efficacy of pilus-based vaccines.

Nevertheless, some studies have reported that pilus antigens have remained common and stable over time. In one recent report, a limited number of pilus antigens in *M. bovis* were reported among Australian strains over the past 20 years (McConnel et al. 2008). Also, when 28 isolates of *M. bovis* from outbreaks of IBK in Argentina, Brazil, and Uruguay were examined for cross-reactivity using rabbit antisera raised against whole cell suspensions, high cross-reactivity indices were reported; one Uruguay strain was cross-reactive with over 75% of the isolates (Conceicao et al. 2003).

The *M. bovis* cytotoxin was recognized in early studies to be conserved among geographically diverse isolates. IBK-affected cattle develop systemic immune responses to cytotoxin (Ostle and Rosenbusch 1985; Hoien-Dalen et al. 1990; Billson et al. 1994). Antihemolysin antibodies against one strain of *M. bovis* neutralized the hemolysin produced by 33 different *M. bovis* strains (Ostle and Rosenbusch 1985). Calves that received vaccination with a partially purified cytotoxin were protected against IBK following experimental challenge with a heterologous *M. bovis* strain (Billson et al. 1994). Such findings have suggested that a cytotoxin vaccine may prevent IBK due to multiple *M. bovis* isolates and could be superior to a pilus-based vaccine.

The homogeneity of *M. bovis* cytotoxins from geographically diverse *M. bovis* was recently demonstrated. The MbxA deduced amino acid sequence from California, Washington, and North Carolina, and Georgia isolates of *M. bovis* and reference strains of *M. bovis* from the National Animal Disease Laboratory, Ames, IA, United States all encoded a nearly identical 927 amino acid protein (Angelos and Ball 2007b). Two *M. bovis* isolates exhibited two amino acid changes relative to all other MbxA deduced amino acid sequences; however, rabbit antiserum against the common carboxy terminus of MbxA from most strains (amino acids 590-927) neutralized the hemolytic activity of the two divergent isolates. These results support the use of cytotoxin in IBK vaccines to protect against diverse strains of *M. bovis*. Two studies evaluating either the recombinant carboxy terminus of MbxA (Angelos et al. 2004) or a recombinant conserved pilin-carboxy terminus of MbxA against naturally occurring IBK (Angelos et al. 2007b) suggest potential for the use of subunit antigens in vaccines against IBK; it is likely that additional antigens will be necessary in these vaccines to provide greater protection against IBK.

CONCLUSIONS

Ongoing research in the area of IBK and its prevention continues to yield new insights into the mechanisms of Moraxella pathogenesis. As additional potentially pathogenic factors are discovered and characterized, novel antigens are identified that might be suitable vaccinal antigens to prevent IBK. While the majority of research into IBK has focused on *M. bovis*, the discovery of *M. bovoculi* in the eyes of cattle with IBK and the identification of the *M. bovoculi* RTX operon suggest that bacteria other than *M. bovis* may play important roles in IBK pathogenesis. Koch's postulates have not been established for *M. bovoculi* and IBK; however, anecdotal reports that vaccination against *M. bovoculi* can prevent IBK when *M. bovis* vaccines are not effective exist. Such reports warrant continued investigations into IBK and its possible causes. These efforts may one day lead to reduced IBK in cattle populations and therefore a reduction in the negative effects it has on individual cattle, animal welfare, and the cattle industry.

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25
Campylobacter and *Helicobacter*

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CAMPYLOBACTER

Organisms in what is now the genus *Campylobacter* were first described in 1880 by Theodore Escherich and were first recognized as etiological agents of abortion in sheep nearly a century ago (McFaydean and Stockman 1913). *Campylobacter* is a curved (1.5–5 µ), gram-negative rod with darting motility. There are currently 14 recognized species (Snelling et al. 2005). The organism is thermo-tolerant, micro-aerophilic, very fastidious, and survives in the intestine of wild and domestic animals. The primary risk factors for acquisition of the organism are direct contact with infected hosts, consumption of animal products, or contact with animal wastes in surface waters.

Campylobacter spp. are the primary cause of ovine abortion in the United States and worldwide, with an average rate of 23.2% in affected flocks (Sahin et al. 2008). A minority of abortions in cattle, goats, pigs, and horses are due to infection with *Campylobacter* spp. (Skirrow and Benjamin 1981). Ewes are exposed to aborted fetuses, placenta, and uterine discharges when an abortion storm begins within a flock (Thompson and Blaser 2000). They show no initial clinical signs until the fetus is aborted, but can occasionally die due to uterine infection and septicemia if the fetus dies and is retained *in utero* (Skirrow 1994). Lesions in aborting ewes include swollen caruncles with exudate, thickened uterine walls, and placentas with mottled, swollen cotyledons (Hedstrom et al. 1987). Aborted fetuses are usually autolyzed, with fluid in the abdomen and thorax. Minor lesions include liver necrosis, suppurative bronchopneumonia, and a moderate neutrophilic infiltrate. *Campylobacter* can be cultured in large numbers from aborted placentas.

Campylobacter fetus ss *fetus* has traditionally been the major species associated with ovine abortion, but recently *Campylobacter jejuni* has also been involved with infectious ungulate sterility and abortion. Results of a study in the United States from 1980 to 1993 demonstrated *C. fetus* ss *fetus* in the majority of abortions during the first 8 years, with *C. jejuni* becoming the dominant isolate in the latter years of the study (Kirkbride 1993). Serotyping demonstrated heterogeneity in *Campylobacter* isolates from abortions on different farms and in different regions, but also demonstrated the presence of specific clones of both *C. fetus* ss *fetus* and *C. jejuni* (Mannering et al. 2006; Sahin et al. 2008). In the United States, chlortetracycline or tetracycline in feed is the treatment of choice for *Campylobacter*-mediated ovine abortion in sheep, but clinical failures of tetracycline treatment to prevent or control abortion storms has been reported.

Campylobacter fetus ss *fetus* is similar to other *Campylobacter* species in its requirement for micro-aerophilic growth conditions. It can be differentiated from *C. jejuni* by susceptibility to cephalothin, better growth at 37°C than 42°C, and by polymerase chain reaction (PCR). *C. fetus* ss *fetus* produces a loosely attached capsular envelope, referred to as the S-layer, which is composed of proteins (SLPs) ranging in molecular weight from 97 to 149 kDa. It is the major virulence factor of *C. fetus* ss *fetus* and

* *Campylobacter*
† *Helicobacter*

appears essential for colonization and/or translocation to the placenta. S-layer proteins also protect against phagocytosis and render the microorganism resistant to serum killing by impairing host C3b binding (Grogono-Thomas et al. 2000). The SLPs are encoded by five to nine *sap*A homologs located in a 53.8-kb chromosomal region. *C. fetus* ss *fetus* usually produces one predominant SLP when cultivated in the laboratory, although variants may be expressed by subpopulations. In the host, each *sap*A homolog is thought to reciprocally recombine with the others, creating new homologs. The new SLPs allow evasion of the host immune response.

Sheep are exposed to *C. jejuni,* as with *C. fetus* ss *fetus*, from the environment or by direct contact with an infected host. Sahin et al. (2008) recently demonstrated *C. jejuni* in 41 of 46 ovine abortion cases submitted to the Iowa Veterinary Diagnostic Laboratory. In addition, 33 of the *C. jejuni* isolates were of the same pulsed-field gel electrophoresis pattern, indicating that the same clone was responsible for the majority of cases, even though submitted from different farms and at different times.

Mucin 2 (MUC2) is the most abundant secreted mucin in the human intestine; it is a major chemoattractant for *C. jejuni*, and the bacterium binds to it (Tu et al. 2008). MUC2 is an environmental cue, exposure to which causes upregulation of 20 genes encoding virulence factors.

Campylobacter jejuni is thermophilic and is cultivated under micro-aerophilic conditions. It can be distinguished from other *Campylobacter* spp. through its hydrolysis of hippurate, or by PCR. A major virulence attribute of *C. jejuni* is its ability to bind to and invade the intestinal epithelium (Babakhani and Joens 1993; Babakhani et al. 1993; Szymanski et al. 1995), which is of paramount importance in induction of abortion. Constitutively expressed proteins of all *C. jejuni* strains mediate epithelial binding. These proteins include a fibronectin-binding protein that attaches to the intracellular matrix (Konkel et al. 1997), a lipoprotein that binds to heat-shock proteins (Jin et al. 2001), Peb1A, which is a periplasmic ABC-binding protein that binds aspartate and glutamate (Pei et al. 1998), and CapA and CapB (Ashgar et al. 2007), autotransporter proteins that mediate an association with intestinal epithelial cells. Minor adhesins involved in epithelial cell binding are lipopolysaccahride and flagella (Kervella et al. 1993). Binding of *C. jejuni* to intestinal epithelial cells initiates events leading to invasion and subsequent disease. Adherence factors are constitutively expressed, but host cell invasion is strain-dependent (Konkel and Joens 1989), with clinical isolates invading at a higher frequency than *C. jejuni* from the environment (Newell et al. 1985). Internalization of *C. jejuni* is by directed endocytosis and involves type III secretion. CiaB proteins synthesized and secreted upon contact are translocated into host cell cytoplasm, which signals the engulfment of attached *C. jejuni* (Konkel et al. 1999). It can then be found in host vacuoles, which merge over 72 h, with exponential growth of the bacterium (Konkel and Ciepak 1992). Increased cell numbers result in epithelial cell lysis and translocation of *C. jejuni* into deeper tissue, such as the intestinal lamina propria and submucosa (Babakhani et al. 1993). In deeper tissue, inflammatory cells take up invading bacteria, with dissemination to other organs.

Survival and dissemination of *C. jejuni* depends on strain characteristics and resistance to antigen processing. Inside the macrophage, *C. jejuni* is exposed to a variety of killing mechanisms, including reactive oxygen species generated by the respiratory burst (De Melo et al. 1989; Kiehbauch et al. 1985). These include superoxide, hydrogen peroxide, and halogenated oxygen molecules. *C. jejuni* circumvents the effects of hydrogen peroxide in the macrophage through production of catalase. Day et al. (2000) demonstrated that a *C. jejuni* catalase-negative mutant was killed within murine peritoneal macrophages within 24 h, whereas wild-type and complemented strains remained viable in high numbers.

Toxins can play a role in fetal death after *C. jejuni* disseminates to the uterus. *C. jejuni* produces two toxins, one of which is a phospholipase encoded by *pld*A. Ziprin et al. (2001) found that mutation of this gene prevented colonization of the ceca of inoculated birds. In contrast, *C. jejuni* was recovered from chicks inoculated with the parent strain. *C. jejuni* also produces a cytolethal distending toxin (CDT) that causes DNA damage, invoking DNA damage checkpoint pathways, and arresting the cells in G_1 or G_2 and leading to necrosis. Assays to determine the effect of a *cdt*ABC mutation revealed no significant difference in adhesion of wild type and mutants to HeLa and HD-11 cells. All mutants had >10-fold decreased ability to invade HeLa cells, but there was no significant difference for HD-11 cells. Studies to determine the ability of mutants to colonize birds

(Biswas et al. 2006) revealed no change in colonization rate between isogenic mutants and the parent strain. These data indicate that the cytotoxin plays no part in adherence to human or avian cells, but it may have a role in invasion and/or survival in ovine cells, which appear to be more susceptible to *C. jejuni* internalization.

HELICOBACTER

Since description of *Helicobacter pylori* as an infectious cause of chronic active gastritis, the number of species in the genus has rapidly expanded. Today, >35 non-*H. pylori* helicobacters have been described in a wide variety of animal species, including humans (table 25.1). Based on the main sites of colonization in the gastrointestinal (GI) tract, helicobacters can roughly be divided into the gastric and the enterohepatic species. Sequencing of the stable small RNA gene (*ssrA*) has shown that the gastric and enterohepatic species have different sequences at the two termini of the tag peptide, indicating different working mechanisms in rescuing stalled

Table 25.1. Non-*H. pylori Helicobacter* Species and Their Hosts

Helicobacter species	Animal hosts	Demonstrated in humans
Gastric *Helicobacter* species		
Cand. H. bovis	Cattle	Yes
Cand. H. heilmannii	Dog, cat, wild *Felidae*, nonhuman primates	Yes
H. acinonychis	Cheetah, tiger	No
H. baculiformis	Cat	No
H. bizzozeronii	Dog, cat	Yes
H. cetorum	Whales, dolphins	No
H. cynogastricus	Dog	No
H. felis	Dog, cat, (rabbit)	Yes
H. mustelae	Ferret	No
H. salomonis	Dog, cat, (rabbit)	Yes
H. suis	Pig, macaque, mandrill monkey	Yes
Enterohepatic *Helicobacter* species		
Cand. H. colifelis	Cat	No
H. anseris	Goose	No
H. aurati	Hamster	No
H. bilis	Mouse, rat, gerbil, dog, cat, sheep	Yes
H. brantae	Goose	No
H. canadensis	Bird, pig	Yes
H. canis	Dog, cat	Yes
H. cholecystus	Hamster	No
H. cinaedi	Hamster, rat, cat, dog	Yes
H. equorum	Horse	No
H. fennelliae	Dog	Yes
H. ganmani	Mouse	Yes
H. hepaticus	Mouse, gerbil	Yes
H. marmotae	Woodchuck, cat	No
H. mesocricetorum	Hamster	No
H. muridarum	Mouse, rat	No
H. pamatensis	Bird, pig, cat	Yes
H. pullorum	Poultry	Yes
H. rodentium	Mouse, rat	No
H. trogontum	Rat, pig, sheep	No
H. typhlonius	Mouse, rat	No

ribosomes of defective mRNAs (Dong et al. 2007). All gastric *Helicobacter* species produce urease (Pot et al. 2007), whereas most enterohepatic species do not. Urease is thought to play a role in protecting the organisms against gastric acidity by creating a pH-neutral zone around the cell body. Some enterohepatic species (e.g., *Helicobacter hepaticus* and *Helicobacter bilis*) also produce urease, but they do not normally colonize gastric mucosa, possessing other features that allow them to settle in the lower intestinal tract and the liver (Sterzenbach et al. 2007).

Pathogenicity and Zoonotic Potential

During the last two decades, the role of *Helicobacter* species as potential pathogens in both human and veterinary medicine has been investigated intensively, and evidence suggests possible zoonotic transmission of animal helicobacters to humans.

GASTRIC HELICOBACTERS

In 1984, gram-negative bacteria belonging to the genus *Helicobacter* were shown to play an important role in the development of gastritis and stomach ulcers in humans (Marshall and Warren 1984). Later, it also became clear that these organisms were involved in the development of gastric cancer. The best known gastric *Helicobacter* species is *H. pylori*, a slightly curved, micro-aerophilic gram-negative organism that occupies a niche in the stomachs of more than half the world's human population. In developing countries, 70–90% of the population is infected with *H. pylori*, while the prevalence is 25–50% in developed countries (Kusters et al. 2006). This bacterium is considered the primary cause of gastritis and peptic ulceration (Marshall and Warren 1984; Dunn et al. 1997) and is a major risk factor in development of gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Kusters et al. 2006).

Patients with severe gastric complaints are often biopsied in order to establish a diagnosis. *H. pylori* is identified on histology by their typical localization and their characteristic, slightly curved morphology. Two-tenths to 6% of these biopsies, depending on the literature source and the geographical region, contained bacteria with a different, typically long, spiral-shaped morphology (Dent et al. 1987). They were originally referred to as "*Gastrospirillum hominis*" (McNulty et al. 1989), but 16S rDNA sequence analysis led to their placement in the genus *Helicobacter* and the provisionally named "*Helicobacter heilmannii*" Genetic analysis revealed two different types, "*H. heilmannii*" type 1 and "*H. heilmannii*" type 2, which differ by >3% in 16S rDNA sequence. "*H. heilmannii*" cannot be used as a species name, according to taxonomic rules. In fact, it has now become clear that spiral-shaped bacteria in the human stomach, often referred to as "*H. heilmannii*" or "*H. heilmannii*-like organism," are identical to *Helicobacter* species colonizing the stomach of pigs, dogs, and cats. We propose use of "gastric non-*H. pylori* helicobacters" to designate these spiral-shaped bacteria when species-level identification is unavailable.

Gastric Non-*Helicobacter pylori* Helicobacters Associated with Pigs

"*Helicobacter heilmannii*" type 1 is both morphologically and genetically identical to *H. suis* (De Groote et al. 1999a; O'Rourke et al. 2004). This bacterium colonizes the stomach of pigs and was first designated "*Gastrospirillum suis*" (Queiroz et al. 1990). Sequencing of the 16S rRNA gene, fluorescent *in situ* hybridization (FISH), and electron microscopy showed that these organisms belonged to the genus *Helicobacter* and were sufficiently different from all existing species to constitute a new taxon. It could not be thoroughly characterized due to lack of pure cultures, so the organism was described as "*Candidatus* Helicobacter suis" (De Groote et al. 1999a). Recent cultivation of the organism has allowed its description as a species *Helicobacter suis* (Baele et al. 2008a). Infections with *H. suis* have been associated with porcine stomach ulcers (Roosendaal et al. 2000; Choi et al. 2001; Szeredi et al. 2005) and gastritis (Hellemans et al. 2007), but the exact role of *H. suis* in porcine gastric disease remains unclear. Ulceration of the porcine gastric non-glandular mucosa may result in decreased feed intake, decreased daily weight gain, and sudden death (Ayles et al. 1996), thus resulting in significant economic losses.

Gastric non-*Helicobacter pylori* Helicobacters Associated with Dogs and Cats

It is now clear that "*H. heilmannii*" type 2 represents, not a single *Helicobacter* species, but rather a group of species that also colonize the canine and feline gastric mucosa; these include *Helicobacter felis*, *Helicobacter bizzozeronii, Heli-*

cobacter. salomonis, and the uncultured "*Candidatus* H. heilmannii" (Jalava et al. 2001; Trebesius et al. 2001; De Groote et al. 2005; Van den Bulck et al. 2005). Two closely related species, one isolated from a dog and the other from a cat, were described as *Helicobacter cynogastricus* and *Helicobacter baculiformis,* respectively (Van den Bulck et al. 2006; Baele et al. 2008b), but no information is available about the presence of these species in humans.

The majority of *Helicobacter* infections of the canine and feline gastric mucosa are mixed infections. They are present in 67–86% of clinically healthy dogs and in 61–100% of dogs presenting with chronic vomiting (Hwang et al. 2002). The stomachs of 41–100% of cats are infected with spiral-shaped microorganisms, with a slightly higher rate in animals presenting with chronic vomiting (Hwang et al. 2002). The significance of gastric *Helicobacter* species in dogs and cats remains enigmatic, and may be *Helicobacter* species- or even strain-dependent. Most spiral bacteria in the gastric mucosa of dogs and cats have not been identified to species level. It is most likely that each species has its own virulence level, which may be increased due to synergistic effects in mixed infections. There was no pathogenic significance for *H. bizzozeronii* in dogs (Peyrol et al. 1998), whereas canine *H. felis* infection was associated with loss of appetite and vomiting (Haziroglu et al. 1995). Young gnotobiotic dogs experimentally infected with *H. felis* had marked lymphoid hyperplasia in the fundus and the body of the stomach (Lee et al. 1992). However, Simpson et al. (1999) found a similar degree of inflammation in both mature SPF dogs experimentally infected with *H. felis* and in uninfected control dogs. These conflicting observations may be due to differences in virulence among *H. felis* isolates (De Bock et al. 2005) or to host differences such as age and breed. Host genetic background may, indeed, have a decisive influence, as proven in experimental infections with *H. felis* and *H. bizzozeronii* in different mice strains (De Bock et al. 2005). Cats inoculated with *H. felis* presented a pangastric mononuclear infiltration of the mucosa that was equivalent to the inflammatory response in uninfected animals. However, follicular organization of the inflammatory cells was restricted to the infected animals (Simpson et al. 2000; Scanziani et al. 2001).

Gastric non-*Helicobacter pylori* Helicobacters Associated with Cattle

"*Candidatus Helicobacter bovis*" is another non-*H. pylori Helicobacter* species recently detected in the stomach of humans with microscopic evidence of a non-*H. pylori Helicobacter* infection (De Groote et al. 2005). This species has been demonstrated in the pyloric abomasum of calves and adult cattle, but has not been cultivated *in vitro* (De Groote et al. 1999b). It is highly prevalent in bovines (non-published results) and gastric ulcers regularly occur in calves and adult cattle (Dirksen et al. 1997; Ok et al. 2001), but its involvement as an etiologic agent is presently unknown.

Gastric non-*Helicobacter pylori* Helicobacters Associated with Humans

Gastric non-*H. pylori Helicobacter* infection of the human stomach is almost always accompanied by an active chronic gastritis (Stolte et al. 1997; Joo et al. 2007). These lesions give rise to dyspeptic symptoms such as postprandial discomfort, epigastric pain, vomiting, heartburn, and dysphagia (Heilmann and Borchard 1991; Stolte et al. 1997). Gastric non-*H. pylori* helicobacters have also been identified as possible causes of acute gastric and duodenal ulceration (Debongnie et al. 1998). Both types of gastric pathology resolve with clearance of these infections, indicating a possible etiologic role (Goddard et al. 1997). Gastric non-*H. pylori Helicobacter* infection is associated with a less severe gastritis than that in *H. pylori* infection, but the risk of developing MALT lymphoma is higher with the former (1.47–10%) than the latter (0.15–0.66%; Stolte et al. 1997; Morgner et al. 2000; Joo et al. 2007).

Helicobacter suis may be the most prevalent gastric non-*H. pylori Helicobacter* in humans (31%). Gastric non-*H. pylori Helicobacter* species associated with dogs and cats are detected less frequently and *Candidatus H. bovis* only sporadically (De Groote et al. 2005; Van den Bulck et al. 2005).

Gastric non-*Helicobacter pylori* Helicobacters Associated with Other Animal Species

Non-*H. pylori* helicobacters have also been isolated from the stomachs of wild Felidae *(Helicobacter acinonychis, Cand.* H. heilmannii*)*, nonhuman primates (*H. suis, Cand.* H. heilmannii, *H. pylori),* mice (*Helicobacter muridarum),* Syrian hamsters (*Helicobacter aurati),* ferrets *(Helicobacter*

mustelae), whales, and dolphins (*Helicobacter cetorum*). *Helicobacter*-like organisms or their DNA were detected in equine stomachs, but their role in development of gastric ulcers remains speculative, and none have been cultivated (Dimola and Caruso 1999; Scott et al. 2001; Contreras et al. 2007).

ENTEROHEPATIC HELICOBACTERS

A wide variety of enterohepatic *Helicobacter* species colonize the lower intestinal tract of laboratory rodents, such as mice, rats, hamsters, and gerbils. Some, in particular *H. hepaticus* and *H. bilis,* may cause disease. Subclinical infections with enterohepatic *Helicobacter* species may interfere with results of experimental research (Whary and Fox 2006; Ceelen et al. 2007a).

Enterohepatic *Helicobacter* Species Associated with Dogs and Cats

In 1993, identical bacteria were isolated from the feces of clinically healthy dogs and a boy with diarrheal illness, and were classified as *Helicobacter canis* (Stanley et al. 1993). It was also recovered from the liver of a puppy with necrotic hepatitis (Fox et al. 1996), from exotic Bengal cats with endemic diarrhea (Foley et al. 1999), and from clinically healthy cats (Shen et al. 2001). Cats were coinfected with other potential pathogens, including *Campylobacter helveticus* (Foley et al. 1999). Further studies are needed to determine the importance of *H. canis* as a primary enterohepatic pathogen in cats and dogs.

Other enterohepatic *Helicobacter* species in dogs and cats include *H. bilis* (Hänninen et al. 2005), *Helicobacter cinaedi* (Kiehlbauch et al. 1995; Misawa et al. 2002), *Candidatus* H. colifelis (Foley et al. 1998), *Helicobacter marmotae* (Hänninen et al. 2005) and *Helicobacter fennelliae* (Kiehlbauch et al. 1995), but the pathogenic significance of these microorganisms for dogs and cats is not known.

Enterohepatic *Helicobacter* Species Associated with Ruminants, Pigs, and Horses

Kirkbride et al. (1985) isolated a *Helicobacter* strain, later identified as *Helicobacter trogontum* (Dewhirst et al. 2000; Hänninen et al. 2003), from aborted lambs with focal hepatic necrosis. Three of 28 pregnant ewes experimentally inoculated with this agent aborted, and hepatic necrosis was observed in the aborted fetuses (Kirkbride et al. 1985). Other bacteria with similar morphology were isolated from sheep and aborted ovine fetuses. These appeared to be *H. bilis* (Dewhirst et al. 2000; Hänninen et al. 2005).

Enterohepatic *Helicobacter* species have not been described in other ruminants, such as cattle and goats. *Helicobacter pamatensis* (Seymour et al. 1994)*, H. trogontum* (Hänninen et al. 2003), and atypical *Helicobacter canadensis* strains (Inglis et al. 2006) have been isolated from the GI tract or feces of pigs, but their pathogenic significance for this species is unknown.

Helicobacter equorum has been isolated from feces of healthy horses (Moyaert et al. 2007c). The prevalence of this microorganism in various adult horse populations is 0.8–7.9%, but is much higher (66%) in 1- to 6-month-old foals. The species was not detected in stool samples from humans (Moyaert et al. 2007b, 2008). Experimental inoculations revealed that this microorganism colonizes the cecum, colon, and rectum of adult horses without apparent pathology (Moyaert et al. 2007a). The virulence of *H. equorum* for young foals has not been determined.

Avian Enterohepatic *Helicobacter* Species

Helicobacter pullorum has been isolated from the ceca and feces of subclinically infected chickens, as well as from the livers and intestinal contents of laying hens with vibrionic hepatitis (Stanley et al. 1994). Birds inoculated with *H. pullorum* remained clinically healthy, although the bacterium was excreted in their feces throughout the study period (Neubauer and Hess 2006; Ceelen et al. 2007b). Mild lesions were present in the ceca (Ceelen et al. 2007b). *H. pullorum* DNA was also detected in the feces of a diarrheic parakeet (*Psephotus haematogaster*; Ceelen et al. 2006b).

The closely related *H. canadensis* has been mainly associated with geese, and recently also with chickens and Guinea fowl (Fox et al. 2000; Nebbia et al. 2007). *Helicobacter anseris* and *Helicobacter brantae* have been isolated from feces of resident Canada geese (Fox et al. 2006).

Zoonotic Potential of Enterohepatic *Helicobacter* Species

Several enterohepatic *Helicobacter* species have been associated with human disease. In many cases, however, establishment of a causal relationship will

require further characterization, and additional investigations are required to ascertain whether animals indeed constitute potential reservoirs for transmission to humans.

Some enterohepatic *Helicobacter* species that infect laboratory rodents, including *H. hepaticus, H. bilis, H. cinaedi* and *Helicobacter ganmani*, may be considered zoonotic. *H. hepaticus* may play a role in liver carcinogenesis, inflammatory bowel disease, and chronic pancreatitis (Nilsson et al. 2006; Zhang et al. 2006). *H. bilis* has been associated with chronic cholecystitis and biliary duct and gallbladder cancer (Kobayashi et al. 2005). *H. cinaedi* typically causes proctitis, proctocolitis, and enteritis in homosexual men (Totten et al. 1985), but is also associated with bacteremia, particularly in patients with underlying immunosuppression (Matsumoto et al. 2007). These infections can also manifest as cellulitis or septic arthritis (Kiehlbauch et al. 1994; Burman et al. 1995), and the organism has been recovered from the blood and feces of immunocompetent children and heterosexual women without GI symptoms (Vandamme et al. 1990). *H. ganmani* has been reported in pediatric patients with liver disorders (Tolia et al. 2004).

Like *H. cinaedi, H. fennelliae* was first isolated from rectal swabs of homosexuals with chronic diarrhea and proctitis (Fennell et al. 1984; Totten et al. 1985). This bacterium has also been identified in the feces of a dog, but no direct evidence of zoonotic transmission has been reported (Kiehlbauch et al. 1995).

Helicobacter canis has been isolated from the feces of a child with gastroenteritis, from bacteremic humans (Prag et al. 2007), from the stool of a man who developed recurrent cellulitis and bacteremia linked to the recent acquisition of a puppy (Gerrard et al. 2001), and from an immunocompetent patient with bacteremia and multifocal cellulitis (Leemann et al. 2006).

The zoonotic potential of *H. pullorum* is suspected, due to isolation of the organism from patients with enteritis and diarrhea (Stanley et al. 1994; Steinbrueckner et al. 1997). The frequent finding of this microorganism on raw poultry suggests that it may be food-borne. However, it has been difficult to confirm this hypothesis, due to the time gap between food consumption and onset of clinical symptoms and the concomitant difficulty in identifying the source of infection (Tee et al. 2001). Fecal material from 4.3% of human patients with GI disease and from 4% of clinically healthy humans harbors *H. pullorum* DNA, calling into question the presumed association of *H. pullorum* with GI disease (Ceelen et al. 2005). *H. pullorum* may also be involved in pathogenesis of chronic liver and gallbladder disease in humans (Ananieva et al. 2002; Pellicano et al. 2004).

The clinical importance of *H. canadensis* is not fully established, but it has been isolated from feces of patients with enteritis (Fox et al. 2000) and from blood of a 35-year-old man with bacteremia (Tee et al. 2001).

PATHOGENESIS AND VIRULENCE FACTORS

Gastric Helicobacters

The gastric colonization, inflammation, and lesions observed after infection with *Helicobacter* species is the result of a complex interplay between the pathogen and the host immune response, with the gastric epithelial cell caught in the cross-fire. Most of the research concerning *Helicobacter* virulence factors and the evoked host response has been done with *H. pylori.* Much less information is available about gastric non-*H. pylori* helicobacters.

Helicobacter *Colonization*

Long-term colonization of the stomach by *Helicobacter* species is achieved through a combination of bacterial factors, including their helicoidal shape, production of urease to buffer the stomach acid, and flagella-mediated motility. So equipped, helicobacters cross the mucus layer and adhere to mucins or gastric epithelial cells. Moreover, the bacteria must overcome the host immune response.

All gastric *Helicobacter* species produce urease, which hydrolyzes urea to ammonia and CO_2. Ammonia neutralizes stomach HCl, creating a neutral microenvironment surrounding the bacterium. Urease is mainly localized in the cytoplasm, but also becomes associated with the surface of viable bacteria after autolysis of the surrounding bacteria (Marcus and Scott 2001). It consists of two subunits, UreA and UreB. Recently, an *H. pylori* strain unable to produce functional urease was found to colonize and damage the gastric mucosa of Mongolian gerbils (Mine et al. 2005). This seems to contrast with previous results of several studies that have found urease to be essential for colonization. In any case, the role of urease is not limited to

colonization; it also plays a role in *Helicobacter*-induced inflammation.

Motility allows helicobacters to move toward the gastric mucosa (Josenhans et al. 1999; Ottemann and Lowenthal 2002), which has a neutral pH. Gastric helicobacters possess unipolar, bipolar, or peritrichous bundles of 2 to 23 flagella. The flagellum consists of a body, a hook, and a flagellar filament. The filament is composed of flagellin subunits, the predominant FlaA and the minor FlaB. It works as a propeller and is covered by a sheath that is suspected to play a role in acid protection and masking of antigens (Jones et al. 1997). The basal body of the flagellum is embedded in the bacterial cell wall and contains proteins required for rotation and chemotaxis. The hook links the body and the filament.

Adhesion is also believed to be a critical initial step in pathogenesis of gastric *Helicobacter* infections. As noted, *H. pylori* colonizes the mucus layer by adherence to mucins. More than 80% of the *H. pylori* cells are thought to remain in the mucus layer, with only a smaller number adhering to and entering epithelial cells (Hessey et al. 1990; Segal et al. 1996). This seems to be in contrast to other gastric helicobacters. *H. mustelae* adheres firmly to gastric epithelium, with few bacteria lying in the mucus. *H. felis* and *H. bizzozeronii* colonize deep in the gastric glands of experimentally infected gerbils, in close association with parietal cells (De Bock et al. 2006b). Pigs inoculated with stomach homogenates from mice infected with *H. suis* developed follicular gastritis, and *H. suis* cells were found in close contact with mucus-producing epithelial cells and parietal cells; the same has been observed in naturally infected pigs (Hellemans et al. 2007).

Helicobacter pylori and *H. mustelae* adhesion to gastric epithelial cells results in pedestal formation, due to rearrangement of the cytoskeleton (O'Rourke et al. 1992; Segal et al. 1996). Several *H. pylori* adhesins have been described, but there is little consensus on which of them are most important *in vivo*. This controversy arises due to *H. pylori* strain differences and the variable expression of adhesins and host cell receptors. BabA, the best characterized *H. pylori* adhesin, interacts with Lewis b blood group antigens on gastric epithelial cells (Borén et al. 1993; Ilver et al. 1998). Two corresponding genes, *babA1* and *babA2*, have been cloned, but only the latter is functional. Other putative adhesins of *H. pylori* include adherence-associated lipoproteins A and B (AlpA and AlpB), sialic acid-binding adhesin (SabA), HopZ, heat-shock proteins, and lipopolysaccharide (LPS; Mahdavi et al. 2002). Flagella have no apparent direct role in adhesion to gastric epithelial cells, but genes involved in the regulation of *H. pylori* flagella biosynthesis may also regulate the production of an adhesin (Clyne et al. 2000).

Little information is available on putative adhesins of non-*H. pylori* helicobacters. *alpA* and *alpB* are not found in *H. felis* and *H. mustelae* (Odenbreit et al. 1999), and flagella do not play a direct role in promoting adherence of *H. mustelae* to gastric epithelial cells (Clyne et al. 2000).

Gastric helicobacters persist throughout the life of the host in the face of a significant inflammatory response, escaping phagocytosis by macrophages and polymorphonuclear cells (PMNs) and avoiding other aspects of the host response. An infiltration of PMNs is associated with human *H. pylori* infection and gerbil infections with *H. felis* and *H. bizzozeronii* (De Bock et al. 2006a, 2006b), but not in pigs or gerbils experimentally infected with *H. suis*. Inflammation induced by *H. suis* is mainly characterized by lymphocytic infiltration. Catalase produced by *H. pylori* (Hazell et al. 1991) and several non-*H. pylori* helicobacters (*H. felis* [Lee et al. 1988], *H. bizzozeronii* [Hänninen et al. 1996], *H. salomonis*, *H. cynogastricus* [Van den Bulck et al. 2006], *H. baculiformis* [Baele et al. 2008a], and *H. suis* [Baele et al. 2008b]) is involved in bacterial resistance to reactive oxygen species (ROS) released by PMNs. Two other enzymes involved in resistance against oxidative stress are alkylhydroperoxide reductase (Lundström and Bölin 2000) and thiol peroxidase (Wan et al. 1997). The presence of the former was confirmed in *H. felis, H. salomonis,* and *H. acinonychis* (Lundström et al. 2001). It seems to be absent from *H. mustelae*, although the corresponding gene (*AhpC*) is found (Lundström et al. 2001).

Helicobacter pylori and *H. felis* LPS induce only a very weak cytokine response mediated by Toll-like receptor (TLR)4. TLR2 seems to be the dominant innate immune receptor for recognition of *Helicobacter* species. It recognizes bacterial lipoprotein and peptidoglycan (Takeuchi et al. 1999). It is not expressed on the gastric epithelium, so the bacteria escape detection and elimination, as long as no other TLR2-expressing cells such as PMNs infiltrate the gastric mucosa (Mandell et al. 2004). Some authors report that TLR5-expressing epithelial cell

lines detect *H. pylori* flagellin (Smith et al. 2003; Torok et al. 2005), while others have reported evasion of TLR5 by *H. pylori* and *H. felis* flagellin (Lee et al. 2003; Gewirtz et al. 2004; Andersen-Nissen et al. 2005), suggesting a mechanism for long-term persistence.

Infection with *H. pylori* elicits local and systemic IgG and IgA antibodies (Blanchard et al. 1999; Akhiani 2005). However, there remains controversy as to whether local specific antibodies play a role in resistance to *H. pylori* infection. *H. pylori* infection was completely cleared from B-cell-deficient mutant mice in the context of severe gastric inflammation (Akhiani et al. 2004). Conversely, wild-type mice remained colonized and developed only mild gastritis, possibly indicating that antibodies impair gastric inflammation and promote bacterial colonization. The regulatory roles of Th1 and Th2 cells in protection against *Helicobacter* infections are incompletely understood. Both the Th1 (Akhiani et al. 2002) and Th2 responses have been linked to protection. Molecular mimicry by *H. pylori* is another possible mechanism of evasion of the immune response. However, it has not been described in non-*H. pylori* helicobacters.

Helicobacter-*induced Gastric Pathology*

Helicobacter gastritis is characterized by infiltration of PMNs and mononuclear cells. Bacterial urease may be involved in *Helicobacter*-induced inflammation. *H. pylori* neutrophil-activating protein (HP-NAP) induces transendothelial migration of PMNs and ROS production by these cells (Brisslert et al. 2005). *napA*, encoding HP-NAP, is probably present in all *H. pylori* isolates, but protein expression is variable (Evans et al. 1995; Allen 2001). *H. felis* is also a very strong stimulator of human PMN chemotaxis, but it apparently does not stimulate these cells to the oxidative burst response (Hansen et al. 2001).

Levels of IFN-gamma, but not of IL-4 and IL-5, are increased in *H. pylori* and *H. felis*-induced gastritis, indicating a local Th1 type response (Smythies et al. 2000; Eaton et al. 2001). The magnitude of the Th1 response in mice correlates with the severity of the gastric inflammation (Eaton et al. 2001). Mice deficient in production of IL-10, which inhibits synthesis of Th1 cytokines, exhibited more severe gastritis than did wild-type mice (Berg et al. 1998; Eaton et al. 2001), suggesting that a Th1 response correlates with severe gastric pathology.

Helicobacter pylori producing the cytotoxin-associated protein (CagA) and carrying the *cag* pathogenicity island (*cag* PAI) are associated with more severe gastritis and a higher risk for development of gastric ulcers or cancer than are CagA-negative strains (Kusters et al. 2006). CagA-positive strains of *H. pylori* also induce apoptosis of rat parietal cells, while CagA-negative strains or *cagE* knockout mutants do not (Neu et al. 2002). In addition, vacuolating toxin (VacA) also plays an important role in development of vacuoles in and induction of apoptosis of epithelial cells (Kusters et al. 2006). Homologous genes have not been found in gastric non-*H. pylori* helicobacters, but *H. felis,* in particular, induces extensive apoptosis and necrosis of parietal cells of experimentally infected gerbils (De Bock et al. 2006a). *H. felis* and *H. suis* induce apoptosis in murine gastric epithelial GSM06 line cells and in human gastric adenocarcinoma AGS line cells (unpublished results). Gamma-glutamyl-transpeptidase, associated with induction of apoptosis in gastric epithelial cells and inhibition of T-cell proliferation by *H. pylori* (Shibayama et al. 2003; Kim et al. 2007; Schmees et al. 2007), is also produced by gastric non-*H. pylori Helicobacter* species.

Long-term experimental infections of mice with human- and primate-derived *H. felis*, *Candidatus* H. heilmannii, and *H. suis* induce MALT lymphoma (Nakamura et al. 2007; Nishikawa et al. 2007). *H. suis* infection induced gastric MALT lymphomas in nearly 100% of C57BL/6 mice by 6 months post-infection, and this was accompanied by parietal cell damage (Nakamura et al. 2007). Up to 25% of BALB/c mice infected with *H. suis* developed MALT lymphoma after 24 months, in contrast to lack of lymphomas in *H. pylori*-infected mice.

As noted, *H. suis* has been associated with ulcers of the non-glandular part of the stomach (Roosendaal et al. 2000; Choi et al. 2001), although the exact role of *H. suis* in porcine gastric pathology remains to be elucidated. Indeed, the work of several authors did not confirm this association (Szeredi et al. 2005). These discrepancies might be due to differences in laboratory techniques for demonstration of helicobacters, different sampling practices, or differences in virulence among *H. suis* strains. In any case, all pigs inoculated intragastrically with *H. suis* in our recent study developed hyperkeratosis and ulceration of the gastric non-glandular mucosa, whereas sham-inoculated control pigs did not (unpublished results). After experimental infection, *H. suis* mainly

colonizes the antrum and the fundic gland zone and, to a lesser extent, the cardiac gland zone (Hellemans et al. 2007). *H. suis* DNA was also detected in the pars esophagea (Roosendaal et al. 2000), but bacteria were not detected by microscopic examination of the non-glandular part of the stomach (Hellemans et al. 2007). The pH range in the non-glandular region and cardiac gland zone, together representing almost 50% of the porcine stomach, is 5-7, due to the presence of saliva and cardiac gland bicarbonate secretions (Höller 1970). The distal compartment, composed of fundic and pyloric glands, ensures postprandial pepsin digestive enzymatic activity through acid secretion. Infection with *H. suis* may result in secretion of excessive amounts of gastric acid, leading to increased contact of the non-glandular part of the stomach with HCl. These microorganisms are found in close contact with parietal cells in the fundic gland region of pigs experimentally or naturally infected with *H. suis,* possibly indicating that the bacterium may impact these HCl-producing cells (Hellemans et al. 2007). An *H. suis* infection results in gastritis, which is mainly localized in the antrum (Hellemans et al. 2007). Increased acid production in human *H. pylori* infections has been associated with antral-predominant gastritis (Kusters et al. 2006). Porcine *H. suis* infection results in an increased number of gastrin-producing cells and a decreased number of somatostatin-producing cells (Sapierzynski et al. 2007). Gastrin stimulates and somatostatin inhibits the secretion of HCl by parietal cells, perhaps also resulting in excessive acid production. However, Silva et al. (2002) did not find increased postprandial serum gastrin concentrations in pigs with ulceration of the pars esophagea. Non-glandular stomach ulceration in pigs is clearly a disease of multifactorial etiology, including dietary and stress factors. Small particle size of feed, interruption of feed intake, and the presence of highly fermentable carbohydrates in the diet promote ulceration (Friendship 2006).

Enterohepatic Helicobacters

Pathogenesis of most enterohepatic *Helicobacter* infections continues to be poorly understood, and it is mainly the interactions of *H. hepaticus* and *H. bilis* with their rodent hosts that have been examined. Cytolethal distending toxin (CDT), which causes progressive enlargement and eventual death of Chinese hamster ovary and HeLa cells, was first described in an enteropathogenic strain of *Escherichia coli*. Toxins belonging to this group were subsequently identified in other diarrheagenic bacteria, including *Campylobacter* spp., *Shigella* spp., and *Salmonella enterica* serovar Typhi. *CdtA, CdtB* and *CdtC*, encoded by a cluster of three adjacent genes, are required for toxic activity. The effects involve cellular distension, cytoskeletal abnormalities, G_1, S, or G_2 cell cycle arrest, and cytolethality in cell lines treated with bacterial culture supernatant or sonicate of bacteria producing the toxin (Ceelen et al. 2006a). CdtB homologs have been identified in *H. bilis, H. canis, H. hepaticus, H. cinaedi,* and *H. pullorum* (Chien et al. 2000; Taylor et al. 2003; Ceelen et al. 2006c). Isolates of *H. pullorum* from birds (10 strains) and humans (three strains) were PCR-positive for *cdtB*, and CDT activity of one human strain was demonstrated in Hep-2 cells (Ceelen et al. 2006c).

Granulating cytotoxin (GCT) has been described in *H. hepaticus* (Taylor et al. 1995). Its cytotoxic activity, demonstrated in CCL9.1 mouse liver line cells, is characterized by appearance of cytoplasmic granules in intoxicated cells. The role of this toxin in pathogenesis is not known. Recently, Young et al. (2004) stated that GCT might represent the previously described CDT, but lack of effect of GCT on HeLa cells, which were used to demonstrate CDT activity, contradicts this statement (Young et al. 2000).

Urease has been mentioned as an important virulence factor for gastric helicobacters that inhabit a highly acidic environment. The function of this enzyme in non-gastric *Helicobacter* species such as *H. hepaticus* and *H. bilis* remains unclear, because these species inhabit a biological niche where the pH is nearly neutral. Possible roles include improving survival during passage through the stomach, as described for *Yersinia enterocolitica*, and producing ammonia as a source of nitrogen for protein biosynthesis. Moreover, urease activity could significantly contribute to tissue damage, since ammonia damages host cells and urease itself stimulates phagocyte chemotaxis, activates immune cells, and induces the production of cytokines (Beckwith et al. 2001).

Certain enterohepatic *Helicobacter* species use H_2, produced as a fermentation by-product in the gut, as an energy substrate through a respiratory hydrogenase. More specifically, hydrogenase oxidizes molecular hydrogen to yield protons and electrons. The low-potential electrons enter the electron transport chain and help in energy conservation. H_2

utilization might be required for energy production to permit growth and maintain virulence in several pathogenic bacteria, including *H. hepaticus* (Maier et al. 2003; Maier 2005). A *H. hepaticus* hydrogenase deletion mutant was unaffected by the presence of hydrogen, while the wild-type strain had an enhanced growth rate when supplied with H_2. Moreover, the livers of mice inoculated with the wild-type strain exhibited moderate lobular lymphoplasmacytic hepatitis with hepatocytic coagulative necrosis, whereas the hydrogenase mutant caused no lobular inflammation or necrosis (Mehta et al. 2005).

Yanagisawa et al. (2005) demonstrated that *H. pullorum, Helicobacter cholecystus, H. bilis*, and *H. hepaticus* produce matrix metalloproteases (MMPs) upon contact with human colon, liver, and bile duct line cells. MMPs participate in degradation of extracellular matrix, allowing bacteria to interact with target cells. This may play an important role in invasion and progression to cancer by enterohepatic *Helicobacter* species colonizing the hepatobiliary and intestinal tracts.

Catalase protects the organism from the damaging effects of H_2O_2, allowing long-term persistence in the host, and NADPH quinone reductase catalyzes the reduction of quinone to quinols (Hong et al. 2007, 2008). These compounds play a part in oxidative stress resistance in many bacterial species, and homologs exist in *H. hepaticus*. An *H. hepaticus* catalase mutant was more sensitive to high O_2 concentrations and to several oxidative stress reagents than the wild type.

CONCLUSIONS

Several helicobacters colonize the GI tract of domestic animals, sometimes resulting in clinical disease. Some are of zoonotic importance. Animal-associated helicobacters are typically very fastidious microorganisms that are cultivated *in vitro* only with great difficulty, if at all. This has seriously hampered research on pathogenesis. There is clearly a need for further studies on the interactions of these microorganisms with their hosts. Recent successes in isolation of gastric and enterohepatic helicobacters from domestic animals will most probably stimulate such work.

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26
Lawsonia intracellularis

C. J. Gebhart and R. M. C. Guedes

Lawsonia intracellularis is the sole species in the bacterial genus *Lawsonia*. It is the etiologic agent of proliferative enteropathy, an infectious, intestinal hyperplastic disease characterized by thickening of the mucosa of the intestine due to enterocyte proliferation (McOrist and Gebhart 2006; fig. 26.1A). *L. intracellularis* is an obligately intracellular, vibroid-shaped bacterium that is found in the apical cytoplasm of infected enterocytes (fig. 26.1B). This bacterium infects mitotically active enterocytes and prevents these cells from differentiating into mature enterocytes, resulting in enterocyte proliferation *in vivo* (Lawson and Gebhart 2000).

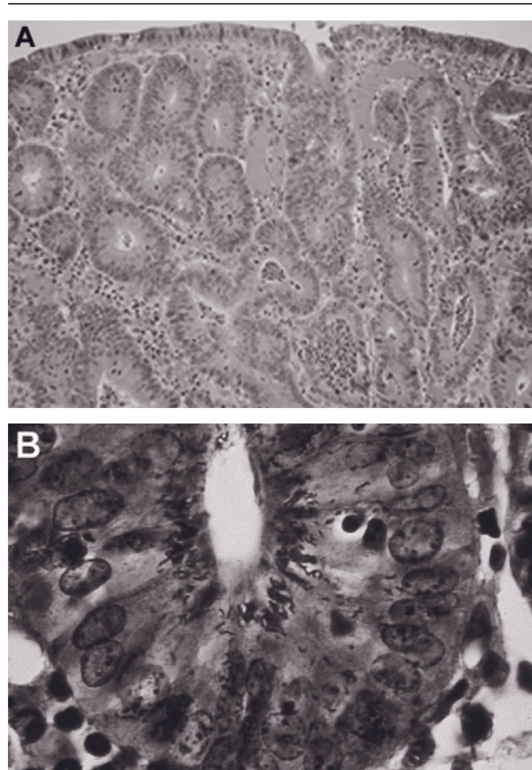

Figure 26.1. Ileal section of a pig experimentally infected with *L. intracellularis*. (A) H & E stain of hyperplastic crypts; (B) silver stain showing numerous vibrio-shaped bacteria in the apical cytoplasm of the proliferating epithelial cells.

Proliferative enteropathy has been reported in numerous and varied animal species (Cooper et al. 1997a; Lemarchand et al. 1997; Cooper and Gebhart 1998; Klein et al. 1999) but has been best described in pigs, hamsters, and horses. Various names have been used, including proliferative enteritis, porcine intestinal adenomatosis, proliferative hemorrhagic enteropathy, ileitis, wet-tail disease, and intestinal adenomatous hyperplasia. The two major clinical forms of proliferative enteropathy in pigs are acute hemorrhagic diarrhea (sudden death of replacement animals and pigs close to market age, known as proliferative hemorrhagic enteropathy) and chronic mild diarrhea and reduced performance in growing pigs, known as porcine intestinal adenomatosis (Lawson and Gebhart 2000; McOrist and Gebhart 2006). Affected hamsters have lesions comparable to the chronic form of proliferative enteropathy in pigs, with the exception that pyogranulomatous inflammation is noted in the late stages of disease (Jacoby et al. 1975). Pathological lesions in horses

range from multifocal to confluent regions of mucosal hyperplasia, resulting in a protein-losing enteropathy (Lavoie and Drolet 2007).

Although the disease in pigs was first reported in 1931 (Biester and Schwarte 1931), it was not until the early 1970s that Lawson's research group in the United Kingdom began studying field outbreaks of proliferative enteropathy (Lawson and Gebhart 2000). The early description of the disease was based on gross and histologic features followed by demonstration of its reproducibility (Biester and Schwarte 1931). Rowland et al. (1973) detected intracellular bacteria in proliferative lesions by use of immunofluorescence, probing affected tissue with hyperimmune serum from an affected pig. Roberts et al. (1977) demonstrated that lesion material contained the infectious agent by experimentally infecting pigs with homogenates of affected intestine. However, it was not until the 1990s that this intracellular bacterium was isolated from hamsters (Stills 1991), pigs (Lawson et al. 1993), and horses (Al-Ghamdi 2003), and cultivated *in vitro*. The disease was then reproduced in pigs (McOrist et al. 1993) and horses (Al-Ghamdi 2003) with pure cultures. The causative intracellular agent of proliferative enteropathy was determined to be of a new genus and species (Gebhart et al. 1993) and was named *Lawsonia intracellularis* in honor of Dr. Lawson (McOrist et al. 1995a).

CHARACTERISTICS OF *LAWSONIA INTRACELLULARIS*

Lawsonia intracellularis is a gram-negative, curved, or vibroid-shaped rod that is 1.25–1.75 μm in length and 0.25–0.43 μm in width. The bacterium has a trilaminar outer envelope, which is frequently separated from the cytoplasmic membrane by an electron-lucent zone. No fimbriae or spores have been detected. Extracellular, cell-culture-grown isolates have a long, single, unipolar flagellum (Lawson and Gebhart 2000; fig. 26.2), and the organism has a darting motility *in vitro* upon escape from infected enterocytes. *L. intracellularis* divides transversely by septation, and organisms are located free within the apical cytoplasm of infected enterocytes (Gebhart et al. 1993).

Strict environmental conditions are required for cultivation of *L. intracellularis in vitro*. Cell-culture systems were used in early attempts to isolate the bacterium due to its intracellular location *in vivo* (fig. 26.3). Growth of *L. intracellularis in vitro* requires dividing eukaryotic cells and an atmosphere of 82.2% nitrogen, 8.8% carbon dioxide, and 8% oxygen (Lawson et al. 1993). Cultivation of the bacterium in these cells continues until high levels of bacteria (>90% infected eukaryotic cells) are achieved, which usually takes 5–7 days (Lawson and Gebhart 2000).

Since it is not possible to cultivate *L. intracellularis* in conventional bacteriological media, the final classification was done by molecular taxonomic methods. Gebhart et al. (1993), using 16S rDNA sequence analysis, showed that sequences obtained from organisms purified, without cultivation, from the ileal mucosa of multiple pigs were 91% similar to those of *Desulfovibrio desulfuricans*. However, the physiological and biological features of the organism were unique enough for it to be

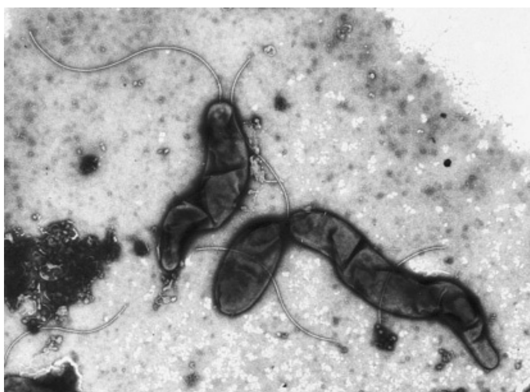

Figure 26.2. Transmission electron microscopy photograph of *L. intracellularis* cells depicting the presence of a single polar flagellum.

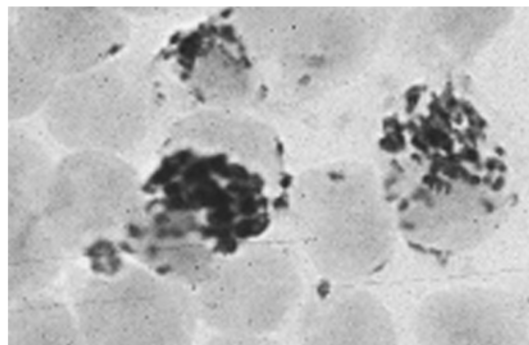

Figure 26.3. Cell culture of intestinal 407 cells infected with *L. intracellularis.* Only a few cells are heavily infected. Immunoperoxidase stain using *L. intracellularis*-specific monoclonal antibody.

considered a new genus. Its closest genetic relative is *Bilophila wadsworthia* (92% 16S rDNA similarity), a free-living anaerobic human pathogen (Sapico et al. 1994). *L. intracellularis* is classified in the delta subdivision of Proteobacteria (Gebhart et al. 1993). Although this bacterium replicates within eukaryotic cells, similar to *Rickettsiae* and *Chlamydiae*, it is taxonomically distinct from these and other intracellular pathogens (McOrist et al. 1995a; Dale et al. 1998; Schmitz-Esser et al. 2008). This intracellular bacterium, previously known as a *Campylobacter*-like organism, *Ileal symbiont intracellularis*, and *Ileobacter intracellularis*, was established in a new genus as *L. intracellularis* (McOrist et al. 1995a).

Isolates of intracellular bacteria from lesions of a variety of animal species have >98% 16S rDNA similarity to pig isolates (Peace et al. 1994; Cooper 1996; Cooper et al. 1997b; Cooper and Gebhart 1998). Phenotypical characterization, by immunoblotting of outer membrane proteins, and conventional molecular characterization methods of the *L. intracellularis* genome demonstrate only minor differences among isolates from different host species and no strain differences within host species (Al-Ghamdi 2003; Guedes and Gebhart 2003c). More recently, multiple loci variable number of tandem repeat (VNTR) sequences have been identified in the genome of *L. intracellularis*. Results of VNTR typing show that samples from epidemiologically unrelated outbreaks of porcine or equine proliferative enteropathy had unique VNTR profiles. In contrast, isolates from the same outbreak shared identical VNTR profiles, a finding consistent with the notion that VNTR profiles are stable over short-time intervals and are likely to be of considerable utility in epidemiological analyses of proliferative enteropathy outbreaks.

SOURCES OF *LAWSONIA INTRACELLULARIS*

Proliferative enteropathy has been reported in a broad range of hosts, including pigs, nonhuman primates, hamsters, rabbits, rats, guinea pigs, foals, sheep, white-tailed deer, ferrets, arctic foxes, dogs, and certain birds (Cooper and Gebhart 1998; Lawson and Gebhart 2000). *L. intracellularis* infection has been established experimentally in mice deficient in interferon-gamma receptors (Smith et al. 2000) and has occurred spontaneously in conventional mice (Guedes 2008). There are no reports of proliferative enteropathy in human beings, but recent reports of the disease in nonhuman primates suggest that human cases may be found as diagnostic methods improve.

The source of infection for these animal species has not been determined. It may be endemic in certain species or it may be present in the environment. Free-living animals serve as the reservoir host and are involved in the transmission of *L. intracellularis* between affected animals or from the natural environment (Pusterla et al. 2008). Species-to-species transmission has been documented. Pig isolates infect hamsters (McOrist and Lawson 1987), mice (Smith et al. 2000), and horses (Al-Ghamdi 2003), and a horse isolate infects hamsters (Cooper 1996). However, cross-species transmission of proliferative enteropathy requires immunosuppression of the target host and results in subclinical, mild infections and, therefore, some host specificity for infection seems to exist.

Feces from infected pigs may be the source of new infections for susceptible animals (McOrist and Gebhart 2006), and pig-to-pig contact is an important route of transmission (Winkelman 1996). Other possible mechanisms of transmission include mechanical vectors, such as rubber boots, and biological vectors, such as mice, small birds, and insects.

Isolation or re-isolation of viable organisms from diseased intestines is not easily achieved due to the difficulty in extraction, growth, and maintenance of *L. intracellularis in vitro*. Therefore, information about the survival and resistance of the organism in the environment is scarce. Intestinal colonization of pigs by *L. intracellularis* was detected after oral inoculation with feces from positive animals, which had been stored for up to 2 weeks at 5ºC or 15ºC (Collins et al. 2000). Pure cultures were fully susceptible to a quaternary ammonium disinfectant, glutaraldehyde, and formaldehyde, less so to povidone-iodine, and not at all to 1% potassium peroxymonosulfate or a phenolic mixture.

VIRULENCE FACTORS

The virulence factors of *L. intracellularis* are not well characterized. Its major pathogenic mechanism is invasion of enterocytes and induction of hyperplasia in these cells (Lawson and Gebhart 2000). Generally, no significant inflammatory response occurs, and the infection remains localized in enterocytes.

Attachment and entry into immature epithelial cells occur at the apical surface. Specific adhesins or receptors for *L. intracellularis* have not been characterized, but attachment and entry appear to require specific bacterium-host cell interaction (McOrist et al. 1995b). The process of invasion does not depend on *L. intracellularis* viability as eukaryotic cells internalize formalin-fixed organisms (Lawson et al. 1995). Invasion is significantly reduced by blockage of cellular metabolism and cytoskeleton rearrangement by cytochalasin D; other mechanisms of cell entry may be involved, since many cells become infected despite treatment with cytochalasin D.

L. intracellularis escapes from the membrane-bound vacuole and lies free in the cytoplasm. *Shigella*, *Listeria*, *Rickettsia* spp., and *Clostridium piliforme* behave similarly, and phagosomal escape is facilitated by lytic toxins (cytolysins or hemolysins). Cytolytic (hemolytic) activity was observed in *L. intracellularis* infection *in vitro* (Hannigan 1997). A protein hemolysin may be involved in attachment and invasion *in vitro* and *in vivo* by facilitating escape from intracellular vacuoles (McCluskey et al. 2002).

Most studies on the pathogenesis of *L. intracellularis* have been conducted *in vivo* since the cellular proliferation that is characteristic of proliferative enteropathy has not been reproduced *in vitro*. The mechanism by which cell proliferation is induced is unknown. Heavy infection corresponds to a reduction in T cell and B cell numbers *in vivo*. This suggests that the bacterium may modulate the immune response, perhaps through an immunosuppressive mechanism (MacIntyre et al. 2003).

Temporary reduction in apoptosis induced by *L. intracellularis* infection was speculated to be a possible mechanism involved in enterocyte proliferation. Absence of the bacteria has been associated with resumption of apoptotic events in the intestinal mucosa and increased numbers of normal epithelial cells in recovering lesions (McOrist et al. 1996). However, the frequency of apoptotic events in affected and unaffected cases was not compared. Other studies (Guedes 2002) suggest an increase in apoptosis in hyperplastic crypts and villi of pigs with naturally occurring proliferative enteropathy. Caspase-3 immunohistochemical staining was used to show that hyperplastic crypts in the ileum, presumably heavily infected by *L. intracellularis*, had significantly higher numbers of apoptotic cells compared with normal crypts ($P < 0.0002$; Guedes 2002). Thus, it appears that cell proliferation, a characteristic feature of proliferative enteropathy, is not caused by reduction in apoptosis.

Little is known about the genetic basis for the virulence, pathogenesis, or physiology of *L. intracellularis*. Further, the molecular mechanisms for infection and virulence and the epidemiology of this organism in pigs and other species remain undetermined. The whole genome sequence of *L. intracellularis* (Gebhart and Kapur 2002) shows that it contains a small chromosome (1.45 Mb) and three plasmids, generating energy by means of a respiratory metabolism. Sequences in the genome of interest from a virulence standpoint include those homologous to genes encoding proteins involved in flagellar biosynthesis and assembly. Identification of a flagellum in *L. intracellularis* isolates, coupled with the sequences that correspond to genes involved in flagellar assembly, provides a means of developing specific reagents to delineate the role of flagella in virulence and infectivity. Sequence analysis also identified homologs of a membrane-bound *Yersinia* outer protein (Yop; Donnenberg 2000) and of LvrV (Bleves and Cornelis 2000), suggesting that *L. intracellularis* is likely to contain a type III secretion system. This secretion system may be involved in bacterial cell invasion and in the evasion of the host's immune system.

The presence of a gene coding for a nucleotide transport protein similar to *Chlamydiae* and *Rickettsiae* adenosine triphosphate (ATP)/adenosine diphosphate (ADP) translocases suggests that *L. intracellularis* acquired this gene by lateral gene transfer from such other intracellular pathogens (Schmitz-Esser et al. 2008). This finding suggests that *Lawsonia*, phylogenetically related to *Deltaproteobacteria*, more recently adapted to an obligately intracellular lifestyle.

PATHOGENESIS

Overview

Comprehensive studies on lesion development and evolution have been conducted in hamsters (Jacoby 1978; Johnson and Jacoby 1978) and pigs (Guedes 2002). Morphological studies of early lesions in experimentally infected animals indicate that entero-

cyte hyperplasia is directly preceded by the presence of intracellular organisms (Jacoby 1978; Johnson and Jacoby 1978; Guedes 2002). *In vivo*, the onset of hyperplasia associated with proliferative enteropathy follows an increase in the number of intracellular *L. intracellularis* in enterocytes. Likewise, resolution of the lesions is closely related to the disappearance of the intracellular organisms, indicating a correlation between the two events (Lawson and Gebhart 2000). The means by which *L. intracellularis* produces hyperplasia is unknown. No other cytopathologic effects on infected enterocytes are seen *in vivo* or *in vitro*. Inflammation is a factor only in later stage lesions and is not a characteristic of the primary lesion.

Progression of Infection

Studies describing the development and evolution of proliferative lesions have been conducted in hamsters (Jacoby 1978; Johnson and Jacoby 1978) and pigs (McOrist and Gebhart 2006; Guedes 2002). An ultrastructural study (Johnson and Jacoby 1978) using hamsters inoculated with homogenates of affected intestinal mucosa showed that the primary lesion was mucosal hyperplasia, with progressive replacement of mature villous columnar absorptive cells by undifferentiated crypt-type cells. Those undifferentiated cells expanded onto villous walls from their normal location in crypts by day 10 postinfection and reached the tips of the villi by day 14. Aggregates of *L. intracellularis* were observed in the apical cytoplasm of crypt epithelium cells by day 5. Enterocyte hyperplasia could only be detected by day 10 using light microscopic examination of hematoxylin and eosin-stained sections (Jacoby 1978). Enterocyte hyperplasia associated with the presence of *L. intracellularis* was observed for up to 40 days postinoculation in hamsters challenged with intestinal mucosa homogenate from diseased animals (Frisk 1976; Jacoby 1978).

Conventional or gnotobiotic pigs colonized by nonpathogenic enteric flora and inoculated with pure cultures of *L. intracellularis* have consistently developed clinical signs and macroscopic lesions of proliferative enteropathy (Mapother et al. 1987; McOrist et al. 1993, 1996; Smith and McOrist 1997; Guedes and Gebhart 2003a, 2003b). Three weeks following oral inoculation with *L. intracellularis*, intracellular organisms, as well as enterocyte proliferation, were identified in intestinal sections.

Electron microscopic examination of early lesions of proliferative enteropathy in pigs and hamsters reveals that *L. intracellularis* in the crypt lumen associates with the cell membrane and enters the immature epithelial cell via an entry vacuole (McOrist et al. 1989). The vacuole breaks down and bacteria multiply freely within the cytoplasm. These infected epithelial cells continue to undergo mitosis, transmitting the organisms to daughter cells (McOrist et al. 1995b). Eventually, the bacteria are released from cytoplasmic extrusions on the epithelial cells at the villous apices or between crypts. Infection spreads to the entire ileum, distal jejunum, cecum, and colon. M cells may be involved in pathogenesis, since lesions in early stages of infection appear in the Peyer patch area of the intestine (McOrist et al. 1993).

Examination of the genesis of lesions during experimentally produced proliferative enteropathy in pigs revealed *L. intracellularis* antigen in the intestine 3–5 days after inoculation. Microscopic lesions, consisting of enterocyte hyperplasia and reduced numbers of goblet cells, were observed 11 days after inoculation, and initial macroscopic lesions were detected 12 days after inoculation. Positive staining, but no gross or microscopic lesions, was detected on day 29, and pigs euthanized on day 35 postinoculation had no lesions and were negative by immunohistochemical staining. *L. intracellularis* antigen was not detected in any organ other than the intestine, lymph node, and tonsils. It appears that *L. intracellularis* infection is limited to enterocytes and bacterial antigen in the lamina propria, and mesenteric lymph nodes may simply be carried there by macrophages. Infection of enterocytes in the large intestine and rectum occurs later in the course of the disease and, consequently, the infection can be detected later in these locations. It appears that the small intestine is infected first, and the bacteria shed from those sites infect lower levels of the intestine. The tonsils do not appear to play a role in pathogenesis, but tonsillar crypt cells may have *L. intracellularis* antigen in their cytoplasm.

Growth and maintenance of *L. intracellularis in vitro* paved the way toward understanding the mechanisms involved in the bacterial entrance into eukaryotic cells and the evolution of the infection in cell cultures (Lawson et al. 1993). Infection of cell cultures *in vitro* has many of the features of the disease *in vivo*. The entry process is dependent on

eukaryotic cell activity and independent of bacterial viability (Lawson et al. 1995). Inhibition of the internalization process by cytochalasin D suggests that it is actin-dependent (McOrist et al. 1997). Close association between *L. intracellularis* and the cell surfaces of cultured rat (IEC-18) and pig (IPEC-J2) enterocytes was observed 10 min after infection. Bacteria were internalized after 3 h within membrane-bound vacuoles and then released into the cytoplasm with the breakdown and loss of these membrane-bound vacuoles. Multiplication of the bacteria by binary fission free in the cytoplasm was observed 2–6 days after cell-culture infection. Drugs that inhibit cell growth also inhibit multiplication of *L. intracellularis*, indicating that cell division is required for bacterial multiplication (Lawson et al. 1995). Six days after infection, highly infected eukaryotic cells have balloon-like, cytoplasmic protrusions, replete with bacteria that are then released from the cell (McOrist et al. 1995a).

Influence of Environmental Factors

Observations suggest that the intestinal flora may modify the ability of *L. intracellularis* to colonize the intestinal tract and cause proliferative lesions. Germ-free pigs are not susceptible to infection by cell-culture-purified *L. intracellularis* (McOrist et al. 1994). However, pigs exposed to intestinal material from *L. intracellularis*-infected animals develop the disease (McOrist and Lawson 1989). Gnotobiotic pigs inoculated with cultured, normal intestinal flora bacteria along with cell-cultured *L. intracellularis* also develop typical lesions of proliferative enteropathy (McOrist et al. 1994).

Dual infections with *L. intracellularis* and other pathogens in conventional pigs have been described. One pathogen may modify the immune response, and thus predispose the animal to a dual infection.

Diet may influence infection in hamsters (Jacoby and Johnson 1981) and pigs (Boesen et al. 2004). Molbak et al. (2008) used a culture-independent approach based on the terminal restriction fragment length polymorphism technique to demonstrate that nonpelleted diet reduced the relative amount of *L. intracellularis* in the total microbiota of the ileum. However, the number of animals shedding the bacteria in the feces was not influenced. The reduction in the relative amount of *Lawsonia* in the ileum of these pigs might have been related to a change in the intestinal environment, such as lower pH, different microbiota, and/or different intestinal morphology (Molbak et al. 2008).

Lesions

Macroscopic lesions of proliferative enteropathy are usually located in the aboral portion of the small intestine but can also be found in the jejunum, cecum, and proximal colon. Intestines affected by the acute form of porcine proliferative enteropathy are dilated, thickened, and turgid, with a corrugated serosal surface. Formed blood clots are found in the lumen of the ileum (Ward and Winkelman 1990).

Chronically affected pigs usually have intestines with irregular patchy subserosal edema, mainly at the area of mesenteric insertion. The ileal mucosa is thickened with deep folds and with patches of pseudomembrane covering the mucosa. As lesions progress, mucosal destruction can lead to rapid death (Ward and Winkelman 1990). Hypertrophy and thickening of the muscularis mucosa may occur in animals surviving this necrotic enteritis.

Histologically, acute and chronic forms of the disease have similar characteristics. Adenomatous proliferation occurs among epithelial cells in the crypts of Lieberkühn in the small intestine and mucosal glands of the large intestine, in association with the presence of vibrio-shaped intracellular bacteria in the apices of these enterocytes (Rowland and Lawson 1974; Brown et al. 2007). The crypts are elongated, enlarged, and crowded with immature epithelial cells in a highly mitotic state. There is marked reduction or absence of goblet cells in affected areas, and infiltration of inflammatory cells is minimal (McOrist and Gebhart 2006). Intestines affected by the acute form are severely congested, with accumulation of blood in the lumen. Electron microscopic studies of experimentally infected hamsters and pigs (Johnson and Jacoby 1978; Jasni et al. 1994; Guedes 2002) and naturally infected pigs (Rowland and Lawson 1974; Love and Love 1979; Lomax and Glock 1982) have shown that highly infected enterocytes usually have short, irregular microvilli.

The intracellular organisms observed in association with proliferated enterocytes can be detected by histochemical staining techniques, such as Warthin-Starry silver stain (Rowland and Lawson 1974), immunofluorescence (McOrist et al. 1987), immunoperoxidase stains (McOrist et al. 1987; Jensen et al. 1997), and hybridization with DNA probes (Gebhart et al. 1991, 1994; Kroll et al. 2005).

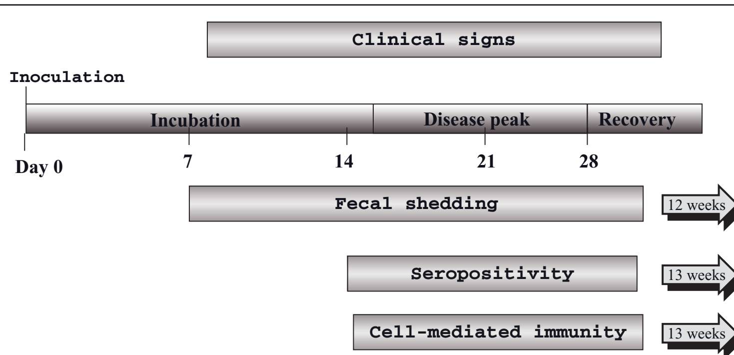

Figure 26.4. Course of proliferative enteropathy in experimentally infected pigs. The onset and duration of clinical signs, fecal shedding, and systemic humoral and cell-mediated immune responses are shown after inoculation of pigs on day 0 with *L. intracellularis*.

Immune Response

IgG was first detected 2 weeks after challenge of 5-week-old pigs with a pure culture of *L. intracellularis*. IgG levels peaked near the end of the third week and then tended to drop (Knittel et al. 1998).

Convalescent pigs may have a degree of immunity to reinoculation (Collins and Love 2007). Animals challenged a second time, after cessation of fecal shedding, were evaluated clinically, and their feces were tested by polymerase chain reaction (PCR) to detect shedding. Animals previously infected did not shed a detectable number of *L. intracellularis* and had no clinical signs. Bacteria in the second challenge inoculum may have been inactivated before entry and colonization of mucosal cells.

The cell-mediated immune response is often an important feature of infections by intracellular organisms. Descriptive immunocytological studies of intestinal tissue sections of pigs affected by both clinical forms of proliferative enteropathy reveal a mild infiltration of cytotoxic T cells, macrophages, and B lymphocytes carrying major histocompatibility complex (MHC) class II structure at the beginning of the cell-mediated immune response (McOrist et al. 1992). Local humoral immunity, in the form of sIgA, is also a relevant defense mechanism against enteropathogenic microorganisms (Lamm et al. 1995). Immunohistochemical studies of intestinal sections of naturally infected pigs demonstrated a large accumulation of IgA in the apical cytoplasm of proliferating enterocytes (Lawson et al. 1979; McOrist et al. 1992). Interferon gamma is produced by peripheral blood mononuclear cells (PBMCs) following specific stimulation (Guedes and Gebhart 2003a), and IgA is detected in intestinal lavages of challenged pigs (Guedes 2002).

Humoral and cell-mediated immune responses were detected 2 weeks after exposure in pigs challenged with a pathogenic isolate (Guedes and Gebhart 2003a; fig. 26.4) and were still detectable in some pigs 13 weeks after exposure. Fecal shedding was initially detected after 1 week and lasted, intermittently, for 12 weeks postexposure. Similarly, interferon gamma played a role in limiting intracellular infections and increased cellular proliferation in experimentally infected mice (Smith et al. 2000). Thus, animals exposed to a pathogenic pure culture isolate of *L. intracellularis* demonstrated long-term shedding and specific immune responses to the organism.

CONCLUSION

L. intracellularis is a unique bacterium, which causes an unusual pathology in infected animals. Hallmarks of proliferative enteropathy are proliferation of enterocytes and the presence of a large number of intracellular bacteria. Limited knowledge of the pathogenesis of *L. intracellularis* suggests that this organism has adopted mechanisms of survival and pathogenesis that are unique among bacterial pathogens (Smith and Lawson 2001). *L.*

intracellularis is genetically unrelated to any of the other intracellular pathogens and, thus, understanding the pathogenic properties of *L. intracellularis* will be an important and intriguing goal as some of these properties may be novel in bacterial pathogenesis.

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27
Gram-negative Anaerobes

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INTRODUCTION

Gram-negative anaerobes represent an extremely diverse group of bacteria, so generalizations about them are difficult. Their study has been hindered by their requirement for anaerobic growth conditions, and often for specialized media; indeed, many species have never been successfully cultured. Most of the anaerobic species encountered in veterinary clinical practice form part of the normal microbiota, and are found in the mouth, or associated with the intestinal, upper respiratory, urinary, or genital tracts. At these sites they are normally present in dense polymicrobial consortia, for example, forming biofilms at the gingival margin. A small number of well-defined species of these bacteria are recognized as important pathogens of animals; these species primarily belong to the genera *Bacteroides, Brachyspira, Dichelobacter, Fusobacterium, Porphyromonas, Prevotella,* and *Treponema*, and are discussed individually in the chapter. These and other anaerobic species are often present in clinical samples in mixed cultures, sometimes together with facultative species. *In vivo* these bacterial mixtures may act synergistically in enhancing infectivity, particularly in the presence of predisposing conditions such as local trauma. Cooperative mechanisms involved in these synergistic interactions may include the supply of energy substrate, essential growth factors, or production of a lower redox potential.

GENERAL BACTERIAL VIRULENCE FACTORS

In the process of establishing infection, and depending on the site involved, pathogenic anaerobic bacteria either need to generate a local anaerobic microenvironment or be able to tolerate oxygen exposure. Anaerobic microenvironments may be created by the activity of bacterial virulence factors such as endotoxin, hemolysin or platelet aggregation factor, or because of synergy with facultative bacteria that consume oxygen. Similarly, oxygen tolerance allows anaerobes to survive in infected tissues until conditions are more conducive for their multiplication and invasion. Many pathogenic anaerobes can grow at low oxygen tensions by utilizing superoxide dismutase, catalase, or NADH oxidase to protect themselves against the toxic effects of oxygen.

In many cases the mechanisms by which gram-negative anaerobes cause disease are ill defined. Until recently, progress has been hampered by difficulties in growing the bacteria, by a lack of genomic information, and by the inability to genetically manipulate many of the species involved. The processes of adherence, colonization, invasion, and tissue destruction generally are facilitated by bacterial structures such as capsules, pili, agglutinins, adhesins, lipopolysaccharide (LPS), and outer membrane proteins; by exotoxins such as enterotoxin, hemolysin, and leukotoxin; and by extracellular

enzymes such as neuraminidase, proteases, DNases, and lipases. Tissue destruction may arise from host immune responses and inflammation that are triggered by specific bacterial products causing the local release of a wide variety of inflammatory cytokines, chemokines, and other mediators. Fermentation products such as lactic acid, butyric acid, and ammonia, which create the characteristic putrid odor of anaerobic infections, also may have inflammatory and cytotoxic effects.

A description of the main pathogenic genera follows. To conserve space, some of the older key references have not been included, but these can be found in previous editions of this chapter.

FUSOBACTERIUM

Fusobacterium necrophorum is a major animal pathogen (Nagaraja et al. 2005). It is one of the 13 species in the genus, all of which produce large amounts of butyric acid as a fermentation product. *Fusobacterium nucleatum, Fusobacterium russi,* and *Fusobacterium canifelinum* are members of the normal oral microbiota of dogs and cats and are frequently isolated from bite wounds (Citron 2002; Conrads et al. 2004). *Fusobacterium equinum* is part of the normal oral microbiota of horses, and is isolated from oral-associated and respiratory diseases of horses (Racklyeft and Love 2000).

Fusobacterium necrophorum

Fusobacterium necrophorum is a normal inhabitant of the mouth, gastrointestinal, and urogenital tracts of animals, and is present in soil. *F. necrophorum* is classified into subspecies *necrophorum* and *funduliforme*, formerly known as biotypes or biovars A and B. These two subspecies have different cellular morphology; colony characteristics; growth patterns in broth (sedimentation or no sedimentation), biochemical (extracellular enzymes), biological (virulence factors and virulence), and molecular characteristics; and frequency of occurrence in infections (Nagaraja et al. 2005; table 27.1). *F. necrophorum* is also a human pathogen, although strains from human infections appear to be distinct from the subsp. *necrophorum* of animal origin, and to more closely resemble subsp. *funduliforme* (Tadepalli et al. 2008c).

Bacterial Virulence Factors

Several structural features, toxins, and enzymes play a role in the pathogenesis of *F. necrophorum* infections. These include leukotoxin, endotoxin, hemolysin, hemagglutinin, the capsule, adhesins or

Table 27.1. Biological and Molecular Characteristics of Subspecies of *Fusobacterium necrophorum*

Characteristics	Subsp. *necrophorum*	Subsp. *funduliforme*	Reference
Biological characteristics			
Virulence in mice, % mortality	92–97	8–10	Lechtenberg et al. 1988
Hemagglutinin titer[a]	15	2.5	Narayanan et al. 1997; Garcia et al. 1999
% lipid A in endotoxic lipopolysaccharide	15%	4%	
Leukotoxin titer	8621	997	
Leukotoxin concentration, μg/ml	694	371	
Molecular characteristics			
rpoB gene[b]	+	+	Aliyu et al. 2004
Hemagglutinin (*haem*) gene	+	–	Narongwanichgarn et al. 2003
Leukotoxin (*lkt*) operon promoter length (bp)	548	337	Zhang et al. 2006

Adapted from Tadepalli et al. 2009.
[a]Reciprocal of the culture dilution that agglutinated a 0.5% chicken erythrocyte suspension.
[b]RNA polymerase.

pili, platelet aggregation factor, dermonecrotic toxin, and extracellular enzymes including proteases, lipases, and DNAses. Hemagglutinins, endotoxic LPS, and leukotoxin are the major virulence factors that participate in the pathogenesis of fusobacterial infections (table 27.2). Hemagglutinin is a low-molecular-weight outer membrane protein (19 kDa) that may play a significant role in adherence to epithelial cells, an initial step in pathogenesis (Kanoe et al. 1998). The concentration and chemical composition of endotoxic LPS varies depending on the subspecies, and the biological activity of LPS of subsp. *necrophorum* is greater than that of subsp. *funduliforme*.

Leukotoxin is a secreted protein that is toxic to ruminant leukocytes, macrophages, hepatocytes, and possibly rumen epithelial cells (Narayanan et al. 2002; Nagaraja et al. 2005). Bovine neutrophils are more susceptible to toxin than peripheral blood mononuclear cells, whereas leukotoxin is less toxic to horse neutrophils and is nontoxic to swine and rabbit neutrophils. This specificity may be a function of the presence of high-affinity receptors, or increased numbers of receptors on the surface of ruminant leukocytes. At high concentrations leukotoxin causes primary cell necrosis. Leukotoxin mediates several potentially important pathogenic mechanisms, including modulation of the host immune system by its toxicity, cellular activation of leukocytes, and apoptosis-mediated killing of phagocytes and immune effector cells (Narayanan et al. 2002). The importance of leukotoxin as a virulence factor in *F. necrophorum* infections is indicated by a correlation between toxin production and ability to induce abscesses in laboratory animals, an inability of leukotoxin-negative strains to induce foot abscesses in cattle following intradermal inoculation, and a relationship between anti-leukotoxin antibody titers and protection against infection in experimental challenge studies (Nagaraja et al. 2005). Carriage of the leukotoxin structural gene *lktA* is almost universal in invasive bovine strains of *F. necrophorum*, although it is less common in non-bovine invasive animal and human strains, in which other virulence determinants must be present (Ludlam et al. 2009).

The leukotoxin operon consists of three genes, *lktB, lktA,* and *lktC*. The *lktA* gene is comprised of 9726 base pairs, and encodes a protein of 3241 amino acids with an overall molecular size of 336 kDa. The LktA protein is considerably larger than leukotoxins produced by other bacteria (Narayanan et al. 2001). The protein may represent a new class of bacterial leukotoxins since it has no sequence similarity to other leukotoxins and is also unusual in that it lacks cysteine. The *lktB* gene consists of 551 codons, yielding a 62-kDa protein that has sequence similarity to HxuB, the hem-hemopexin utilization protein of *Haemophilus influenzae.* The LktB protein has a putative POTRA (for polypeptide-transport-associated) domain at its N-terminus (residues 86-161), which raises the possibility that LktB is involved in the secretion of the LktA leukotoxin. The *lktC* gene encodes a predicted protein of 146 amino acids with a molecular size of 17 kDa, and the protein does not have significant

Table 27.2. Major Virulence Factors of *Fusobacterium necrophorum*

Factor	Characteristics	Mechanism of action	Role in pathogenesis
Hemagglutinin	Outer membrane protein (19-kDa)	Agglutinates erythrocytes of various animals	Mediates attachment to rumen epithelial cells and hepatocytes
Leukotoxin	Extracellular protein (molecular weight 336-kDa)	Cytotoxic to neutrophils, macrophages, hepatocytes, and rumen epithelial cells	Protects against phagocytosis by neutrophils and kupffer cells, damages hepatic parenchyma by the release of cytolytic products
Endotoxin	Cell-wall component, lipopolysaccharide	Cytotoxic and necrotic effects and induces disseminated intravascular coagulation.	Creates anaerobic microenvironment conducive for anaerobic growth.

sequence similarity to proteins currently in the NCBI databases. The biological function of the LktC protein has not been determined. The subspecies *funduliforme lkt* operon is organized identically to the subsp. *necrophorum* operon. Although the overall sequence similarity of the Lkt proteins is high between the two subspecies (87%), the LktB and LktA proteins have significant differences in their N-terminal sequences (Tadepalli et al. 2008b). The activity of the LktA protein expressed in *Escherichia coli* indicates that the other proteins encoded in the leukotoxin operon are not required to produce biologically active toxin. Their role may be in secretion of the toxin across the cytoplasmic and outer membranes of *F. necrophorum*.

All three *lkt* genes appear to be co-transcribed from a promoter contained on the intergenic region upstream of the *lktB* gene (Zhang et al. 2006). The sequence and the size of the intergenic sequence differ in the two subspecies, being 548 bp in subsp. *necrophorum* and 337 bp in subsp. *funduliforme*. The subsp. *funduliforme* promoter activity is weaker than that of subsp. *necrophorum* (Tadepalli et al. 2008b).

Pathogenesis and Diseases

Fusobacterium necrophorum is associated with numerous necrotic disease conditions generally termed "necrobacillosis." These occur in many domestic and wild animals and may involve any part of the body (Nagaraja 1998; Nagaraja et al. 2005). *F. necrophorum* causes a variety of necrotic infections including necrotic stomatitis of calves, lambs, and pigs, footrot in cattle and sheep, pulmonary and hepatic abscesses in cattle and pigs, and jaw abscesses in wild ruminants and marsupials. The organism is a major bovine pathogen. Among *F. necrophorum* infections in cattle, hepatic necrobacillosis (liver abscesses), necrotic laryngitis (calf diphtheria), and interdigital necrobacillosis (footrot) have the most severe economic impacts.

Liver abscesses occur secondary to infection of the rumen wall, and together they form the "rumenitis-liver abscess complex" (Nagaraja and Lechtenberg 2007). Rapid fermentation of grain by rumen microbes and the consequent accumulation of organic acids result in rumen acidosis. Acid-induced rumenitis and damage to the protective surface by coarse objects (e.g., sharp feed particles, hair) predispose the rumen wall to invasion and colonization by *F. necrophorum*. The organism then gains entry into the blood or causes rumen wall abscesses. Bacterial emboli are subsequently shed into the portal circulation where they are filtered by the liver, resulting in infection and abscess formation. Leukotoxin-mediated cytotoxicity to neutrophils could help to prevent phagocytosis and clearing of organisms in the rumen wall and liver. In addition, neutrophil death could lead to parenchymal cell damage, abscess formation, and sequestration, thus maintaining an anaerobic environment in the aerobic hepatic tissue. A decrease in the local neutrophil response due to leukotoxin-mediated death of neutrophils could also enhance colonization of some other opportunistic pathogens, such as *Arcanobacterium pyogenes* in the rumen wall, liver and feet, and *Prevotella melaninogenica* in the feet (Nagaraja 1998).

Calf diphtheria is a necrotic laryngitis occurring in cattle up to 3 years of age, and is characterized by necrosis of the mucosa and underlying tissues of the larynx and adjacent structures. The organism is normally present in the upper respiratory tract, but does not penetrate healthy mucosa; hence, a breach in the mucosal integrity is required for the onset of infection. The infection can be acute or chronic and is noncontagious. In severe cases, cattle may die from aspiration pneumonia.

Interdigital necrobacillosis (footrot, foot abscesses, or foul-in-the-foot) is characterized by acute or subacute necrotizing infection of the skin and of the adjacent underlying soft tissues of the feet. Fecal excretion of *F. necrophorum* is believed to provide the primary source of infection. The infection is the major cause of lameness in dairy and beef cattle in the United States (Nagaraja et al. 2005). The economic impact is from loss in milk production and weight gain. In suppurative infections of the feet, *F. necrophorum* is frequently associated with *A. pyogenes* and *P. melaninogenica.*

Immunity

Serum antibodies against *F. necrophorum* are present in both healthy and infected animals, and therefore the importance of anti-*F. necrophorum* antibodies is unclear. Protection afforded by using a variety of antigenic components, including whole cell cultures, cytoplasmic fractions, LPS, outer membrane proteins, leukotoxins, and culture supernatants has varied from none to significant (Nagaraja and Chengappa 1998). Leukotoxin is

strongly immunogenic and, consistent with it being the primary virulence factor involved in the production of liver abscesses, antibodies directed against it correlate with protection. A vaccine combination of *F. necrophorum* leukotoxoid and *A. pyogenes* bacterin reduced the prevalence and severity of liver abscesses in feedlot cattle (Jones et al. 2004).

Fusobacterium equinum

Fusobacterium isolates from horses, particularly from the respiratory tract, have been assigned to the species *F. equinum* (Dorsch et al. 2001). *F. equinum* is indistinguishable from both of the *F. necrophorum* subspecies based on morphological and biochemical characteristics, but can be distinguished using a polymerase chain reaction (PCR) assay (Tadepalli et al. 2008a). *F. equinum* is a normal inhabitant of the gastrointestinal, respiratory, and genitourinary tracts of horses (Racklyeft and Love 2000). It is an opportunistic pathogen generally associated with abscesses and various necrotic infections in horses, particularly oral, para-oral, and lower respiratory tract infections. In racehorses, bacteriological examination of intra-abdominal and hepatic abscesses has revealed *F. equinum* as the etiological agent in approximately 33% of the cases caused by obligate anaerobes. Little is known about the virulence factors associated with *F. equinum* infections in horses; however, members of the species contain a leukotoxin gene and exhibit leukotoxin activity (Tadepalli et al. 2008a).

BACTEROIDES

The genus *Bacteroides* currently includes numerous species of gram-negative rods, but of these only *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* are common and clinically important. Commensal *Bacteroides* species are present in the large intestine and are considered to be important in the development and maintenance of normal intestinal function and immunological reactivity. Although *B. fragilis* constitutes only about 0.5% of the normal colonic population in humans, it is the most common anaerobe isolated from human clinical samples due to its potent virulence factors (Wexler 2007). Outside the intestinal tract it is associated with abscesses that are mainly intra-abdominal, as well as soft tissue infections and bacteremias.

Bacterial Virulence Factors

Bacteroides species, particularly *B. fragilis,* have numerous features that contribute to adhesion, invasion, and tissue destruction (Wexler 2007). Associated surface features include pili, the capsule, and LPS; toxins include hemolysin and enterotoxin; enzymes with tissue damaging potential include proteases, collagenase, fibrinolysin, neuraminidase, heparinase, chondroitin sulfatase, and glucuronidase. The complex capsular polysaccharide of *B. fragilis* is a major virulence factor in abscess formation. Experimental injection of purified capsular polysaccharide in the absence of organisms induces abscess formation in animal models. Three distinct polysaccharides, PsA, PsB, and PsC, occur in the capsule (Coyne et al. 2000). The repeating units PsA and PsB contain both positively and negatively charged groups, and these may confer a selective advantage in survival in the colon, and the zwitterionic charge motif is a critical feature for abscess formation (Tzianabos et al. 1993).

Strains of *B. fragilis* cause diarrhea in lambs, calves, piglets, foals, infant rabbits, and children. Fragilysin, the *B. fragilis* enterotoxin, is a 20-kDa heat labile polypeptide that causes fluid secretion in intestinal loops, increases bacterial internalization in enterocytes, and modulates epithelial permeability (Obiso et al. 1997). It is a zinc metalloprotease with three different isoforms, and its toxic properties result from its proteolytic activity (Holton 2008). The fragilysin gene encodes a preprotoxin of 44 kDa containing a potential N-terminal signal peptide characteristic of bacterial lipoproteins, and a 22-kDa prosequence. The protoxin is cleaved at an Arg-Ala site to release the 20-kDa extracellular protease (Kling et al. 1997).

PREVOTELLA AND *PORPHYROMONAS*

The genera *Prevotella* and *Porphyromonas* are composed mainly of saccharolytic (*Prevotella*) and asaccharolytic (*Porphyromonas*) pigmented and non-pigmented species that previously were classified as *Bacteroides.* The genus *Prevotella* currently includes eight species (Jousimies-Somer and Summanen 2002), whereas *Porphyromonas* includes 12 pigmented and one non-pigmented species, nine of which are of animal origin. *Porphyromonas gingivalis* is the best known species isolated from humans, and forms part of the "red complex" of

anaerobes that together with *Treponema denticola* and *Tannerella forsythia* is strongly associated with the advanced stages of periodontitis in humans (Holt and Ebersole 2005). By contrast, *Porphyromonas* isolates from the gingival sulcus of various animals are included in the new species *Porphyromonas gulae*. The vitamin K dependency of these genera is met by the presence of other bacteria, so that they are only found in mixed bacterial infections in animals. Their presence in mixed infections means that the disease they cause is likely the sum of the virulence determinants of the different species present.

Bacterial Virulence Factors

Black-pigmented anaerobes produce a variety of virulence factors. These include those that help protect the organisms from oxygen toxicity in less than optimal anaerobic environments (catalase, superoxide dismutase, hemolysin, and LPS), those that protect the organisms from host defenses (capsule, complement degradation, proteases that degrade IgA and IgG, and leukotoxin), those that promote attachment (capsule, pili, hemagglutinins) and others that facilitate invasion and tissue destruction (including collagenase, proteases, RNases, DNases, phospholipase, neuraminidase, hyaluronidase, and heparinase; Holton 2008). *P. gingivalis* also produces a serine protease (HlrA) in response to heat stress that increases cellular invasion and *in vivo* survival (Yuan et al. 2008).

Pathogenesis and Diseases

Prevotella and *Porphyromonas* species have a prominent role in oral disease and in bite wound infections. Periodontal disease affects a wide range of animal species and is the most common oral disease of adult animals; it includes gingivitis, periodontitis, and periodontal abscesses, initiated by bacteria in the dental plaque, and is exacerbated by ineffective host immune responses. *Prevotella* and *Porphyromonas* are frequently isolated from infected dog and cat bite wounds in humans (Talan et al. 1999). *P. melaninogenica* is readily cultured from footrot or foot abscesses of cattle, and may work in synergy with *F. necrophorum* to mediate the infection.

DICHELOBACTER

Dichelobacter nodosus, a large gram-negative rod with enlarged ends and relatively slow growing aerotolerant anaerobe, is an obligate parasite of the feet of cloven-hoofed animals, and the principal causative agent of ovine footrot, an economically important and debilitating disease of sheep and goats (Rood et al. 2005). Footrot is characterized by the separation of the keratinous hoof from the underlying tissue, resulting in severe lameness and loss of body condition. The severity of the disease can vary greatly and is dependent on the nature of the isolate as well as on host and environmental factors.

Bacterial Virulence Factors

Dichelobacter nodosus strains have several potential virulence factors, including Type IV fimbriae, extracellular serine proteases, outer membrane proteins and lipopolysaccharide. Two genomic regions, a virulence related locus (*vrl*) and virulence associated protein (*vap)* region, have been found to be preferentially associated with virulent strains, but the genes present on these regions are not known to encode virulence factors and the role in virulence of any of the genes in these regions is yet to be determined (Billington et al. 1996). Only the fimbriae have been shown to be essential for virulence (Kennan et al. 2001). Several other potential virulence factors were identified in the recently completed genome sequence of strain VCS1703A (Myers et al. 2007), but the role in virulence of these genes is yet to be confirmed.

Fimbriae

Dichelobacter nodosus has Type IV fimbriae, which have a highly conserved hydrophobic domain, an N-methylphenylalanine residue at the N-terminus, located at their poles and associated with twitching motility (Rood et al. 2005). Genome sequencing of a virulent *D. nodosus* isolate identified 21 genes involved or potentially involved in fimbrial biogenesis and 10 genes involved or potentially involved in the regulation of fimbrial biogenesis (Myers et al. 2007). Disruption of the *fimA* gene, which encodes the major fimbrial subunit, resulted in a mutant that was unable to produce fimbriae, was no longer naturally competent for DNA uptake, was unable to undergo twitching motility, and was also unable to secrete any extracellular proteases (Kennan et al. 2001). Virulence testing of two independent *fimA* mutants in a sheep virulence trial showed that these mutants were avirulent, thereby confirming that the

fimbriae are essential for virulence (Kennan et al. 2001). Furthermore, mutation of the pilin-like protein gene *pilE,* or the fimbrial biogenesis genes *fimN, fimO* or *fimP,* resulted in the lack of surface fimbriae, indicating that these genes are essential for fimbrial biogenesis (Han et al. 2007). These mutants also had reduced protease secretion, indicating that an intact fimbrial biogenesis system is required for protease secretion in *D. nodosus* (Han et al. 2007).

To further delineate the role of the fimbriae in virulence, mutants were constructed in *pilT* and *pilU,* orthologs of genes that are reguired for twitching motility in other organisms (Han et al. 2008). These mutants still produced fimbriae, but were unable to undergo twitching motility, while protease secretion was disrupted in the *pilT* mutant, but not the *pilU* mutant. Both mutants were completely avirulent in a sheep virulence trial, thereby demonstrating that it is Type IV fimbriae-mediated twitching motility that is essential for the virulence of *D. nodosus* (Han et al. 2008). The production of these fimbriae is controlled at the level of transcription by a two-component regulatory system PilRS, where PilR is a transcriptional activator that binds specifically to a region upstream of a σ^{54} promoter (Parker et al. 2006).

The fimbriae are highly immunogenic and provide the basis for the classification of *D. nodosus* strains into ten major serogroups designated A to M, and 18 serotypes.

Extracellular Serine Proteases

Dichelobacter nodosus isolates secrete several serine proteases, which are potentially responsible for at least part of the tissue damage observed in diseased animals. As mentioned, the proteases require an intact fimbrial biogenesis system for secretion, and as such appear to be secreted via a type II secretion pathway that utilizes the Type IV fimbrial apparatus (Han et al. 2007). The proteases secreted by virulent isolates have a greater thermostability, and higher elastinolytic and caseinolytic activity than those of benign strains, properties that have been used in diagnostic tests to distinguish benign and virulent isolates (Liu and Yong 1993; Palmer 1993).

Virulent isolates produce two acidic proteases, AprV2 and AprV5, and one basic protease, BprV, whereas the equivalent proteases secreted by benign strains are designated AprB2, AprB5, and BprB (Lilley et al. 1995; Riffkin et al. 1995). All the *D. nodosus* proteases have a similar organization, and are produced with a pre-pro region at the N-terminal of the mature protease and a C-terminal extension, with the active protease produced after cleavage of the precursor (Riffkin et al. 1995). These proteases are all subtilisin-like serine proteases, or subtilases, and have been classified in the B subfamily of this superfamily.

Sequencing of the three protease genes from virulent strains revealed the mature proteases to have 62% amino acid sequence identity across all regions (Riffkin et al. 1995). The benign proteases are very similar to their virulent counterparts; for example, there is only one amino acid sequence difference between the mature AprV2 and AprB2 proteases (Riffkin et al. 1995). The AprV5 and AprB5 enzymes are 99% identical, with only two amino acid changes in the precursor, and BprV and BprB have 96% identity, with 10 amino acid substitutions in the mature protease (Lilley et al. 1995). Recent work has shown AprV2 to be responsible for the elastase activity of virulent isolates and to be required for virulence (R.M. Kennan and J.I. Rood, unpublished results).

Pathogenesis of Footrot

Footrot is a seasonal disease that occurs in warm moist conditions, with a temperature of above 10°C required for transmission. Infected sheep and goats are thought to be the main reservoir of *D. nodosus*, and the anaerobe generally cannot survive for more than a few days outside the feet of ruminants (although it may survive up to 6 weeks on hoof horn clippings; Whittington 1995). Under optimal climatic conditions transmission can occur to other uninfected animals via contaminated pastures and soil, and from transport vehicles contaminated with infected soil and feces (Depiazzi et al. 1998).

Footrot shows a spectrum of severity ranging from benign, which presents as an interdigital dermatitis that does not progress, to virulent, which results in severe underrunning of the horn of the hoof and separation of the hoof from the underlying tissue. New infections always commence at the inter-digital skin, which becomes predisposed to infection after prolonged exposure to wet conditions. These conditions enable invasion of bacteria such as *F. necrophorum* (Zhou et al. 2009), which then provide the necessary conditions to enable *D. nodosus* to establish an infection.

Once the interdigital infection is established, the footrot lesion may progress to the underlying tissue. In the field both *D. nodosus* and *F. necrophorum* are essential for the invasion of the epidermal matrix of the hoof; however, *D. nodosus* alone can cause footrot in experimental settings after damage to the inter-digital skin and applying *D. nodosus* directly to the damaged area with bandages. The primary invader of the epidermal matrix appears to be *D. nodosus*, which is considered essential for the development of clinical footrot. Once a *D. nodosus* infection is established and the process of hoof separation has commenced, an environment is produced in which *F. necrophorum* can flourish and penetrate more deeply into the tissue, causing inflammation and tissue destruction. The extracellular proteases secreted by *D. nodosus* are believed to be critical for the initial separation of the hoof from the underlying tissue. Footrot is accompanied by a foul smell, a gray scum between the epidermis and the horn of the hoof, and by lameness.

Immunity

The primary immune response of sheep to *D. nodosus* infection is dependent on the length and severity of the infection, with the fimbriae inducing the strongest and most specific response (Whittington and Nicholls 1995); however, other *D. nodosus* proteins have also been shown to invoke an antibody response in infected animals (Myers et al. 2007). The fimbriae are the major immunoprotective antigens, but this protection is serogroup-specific. Recombinant fimbrial vaccines have been prepared from overexpression of fimbrial subunits in *Pseudomonas aeruginosa*; however the use of multiple fimbriae in one vaccine leads to a reduced and unpredictable immune response due to antigenic competition (Hunt et al. 1995). Monovalent and bivalent vaccines specific to the infecting serogroups have been used to successfully eradicate virulent footrot (Egerton et al. 2002).

The immune response to infection is variable among different breeds of sheep, and although all breeds are susceptible to footrot, the older breeds of British sheep appear to be less susceptible than Merinos. This natural resistance results in milder lesions, delayed infection and self-curing lesions, and may be paternally inherited and involve alleles of the ovine major histocompatibility complex (MHC) class II region (Escayg et al. 1997).

Diagnosis

Rapid diagnosis of footrot and the differentiation of benign and virulent strains are important for the control and eradication of virulent footrot. Several laboratory tests based on the activity of the secreted proteases have been used to identify virulent strains. A test based on the digestion of the insoluble substrate elastin showed good correlation between the ability to digest elastin in agar plates and the virulence of the isolate. Virulent isolates generally showed digestion of the elastin particles within 6 to 10 days whereas benign strains showed no digestion of the elastin after 21–28 days.

Electrophoretic zymograms were later developed as a rapid method of differentiating benign and virulent isolates, although the existence of complex banding patterns can make this test difficult to interpret (Liu and Yong 1993). The gelatin–gel test is a rapid alternative to the elastase test. It measures the thermostability of secreted proteases after incubation at 67°C. Strains that still show protease activity after heating are regarded as thermostable and this group generally correlates with virulent isolates, whereas strains showing no protease activity after heating for 16 min are regarded as unstable and are generally benign (Liu and Yong 1993; Palmer 1993).

Extensive use of the gelatin-gel test has shown that some strains that are gelatin-gel stable only cause benign footrot (Cheetham et al. 2006). A PCR test based on the *vap*-associated *intA* gene was more reliable for isolates that were stable or equivocal in the gelatin-gel test and it was proposed that use of this *intA* PCR test with the gelatin-gel test should distinguish stable benign strains from stable virulent strains (Cheetham et al. 2006).

The identification of the infecting serotypes is also essential if specific vaccination is to be performed, and PCR methods have been developed to enable the rapid identification of fimbrial serotypes (Dhungyel et al. 2002). These tests, when combined with the gelatin-gel test, allow the rapid diagnosis of the virulence status and serotype of the infecting organisms, enabling more specific treatment and vaccination.

TREPONEMA

Anaerobic *Treponema* species colonize the mouth and the intestinal and genital tracts. As in humans, combinations of different *Treponema* spp. and other

anaerobes in the oral cavity of dogs are associated with periodontitis, a chronic destructive condition of the structures supporting the teeth (Nordhoff et al. 2008b).

Bovine digital dermatitis is an economically significant condition in dairy cows that also is associated with treponemal infection. Affected feet have variable presentation from moist, painful strawberry-like ulcers, to chronic, raised, hairy wart-like lesions. Lesions mainly occur at the rear of the hoof between the bulbs of the heel, and lead to lameness. Combinations of at least three phylogenetic groups of treponemes, some related to human oral treponemes, have been linked with this condition (Nordhoff et al. 2008a; Evans et al. 2009). Ovine digital dermatitis is similarly linked to the presence of *Treponema* species related to those found in cattle with digital dermatitis (Sayers et al. 2009). Lesions begin around the coronary band, the feet are red and swollen, and the disease is aggressive. Currently, little detailed knowledge is available about the epidemiology or pathogenesis of these interesting polymicrobial anaerobic infections in cattle and sheep.

BRACHYSPIRA

The genus *Brachyspira* currently contains seven species of anaerobic intestinal spirochetes that colonize the large intestines of a variety of animal species. Most are 6–8 µm long and 0.3–0.4 µm wide, with 2–4 loose coils. Pathogenic species include *Brachyspira alvinipulli*, *Brachyspira hyodysenteriae*, *Brachyspira intermedia*, and *Brachyspira pilosicoli*. Other species are generally considered to be commensals. *B. intermedia* and *B. alvinipulli* are both pathogens of adult chickens, being associated with reduced egg production and diarrhea (Stephens and Hampson 2001). *B. intermedia* is widespread in adult layer and broiler breeder flocks, whereas *B. alvinipulli* appears to be less common.

Brachyspira hyodysenteriae

Brachyspira hyodysenteriae is the primary etiological agent of swine dysentery (SD), a severe mucohemorrhagic colitis typically affecting grower and finisher pigs. This spirochete also causes a typhlitis in Rheas, and has been recovered from adult chickens and feral and farmed mallard ducks. SD occurs worldwide, and causes considerable economic loss due to poor growth rates and occasional mortality. Recovered carrier pigs and pens and trucks contaminated with infected feces are the main source of infection. Mice and rats may transmit the infection within a piggery.

Bacterial Virulence Factors

Several potential virulence factors have been described for *B. hyodysenteriae*, including chemotaxis, motility, adherence, hemolysin production, and the endotoxic activity of lipooligosaccharide (LOS). Colonization by *B. hyodysenteriae* is enhanced by the presence of other anaerobic species in the large intestine, which contribute to lesion formation. Consistent with this complex interaction, changes in dietary substrate can influence the colonic microbiota, the extent of *B. hyodysenteriae* colonization, and the degree of disease expression (Mølbak et al. 2007). The spirochete shows a chemotactic response toward mucin (Milner and Sellwood 1994), and analysis of the genome sequence from *B. hydysenteriae* strain WA1 has identified large numbers of genes associated with chemotaxis and motility (Bellgard et al. 2009). A corkscrew-like motility that aids penetration of mucus is generated through the activity of the periplasmic flagella. The importance of this motility was demonstrated by the fact that FlaA and FlaB mutants created through allelic exchange showed both reduced motility and reduced colonization in experimentally infected pigs (Kennedy et al. 1997). NADH oxidase activity appears to be important in colonization of the mucosal surface, since the enzyme consumes oxygen and hence reduces potential oxygen toxicity; furthermore, NADH oxidase-negative mutants showed a reduced ability to colonize pigs (Stanton et al. 1999). Three putative hemolysin genes of *B. hyodysenteriae* were originally described (*tlyA, tlyB* and *tlyC*), and hemolysin negative mutants of *B. hyodysenteriae* showed reduced virulence in pigs (Hyatt et al. 1994). A distinct gene (*hlyA*) encoding a beta-hemolytic protein with marked similarities to the native *B. hyodysenteriae* hemolysin was subsequently identified (Hsu et al. 2001). More recently, genomic analysis has identified two additional potential hemolysins (Bellgard et al. 2009). The spirochete also possesses genes for numerous proteases, as well as phospholipases and peptidases that may contribute to local cellular damage; however, only the Sec pathway and no other secretory systems were identified from the genome data. The lipooligosaccharide (LOS) on the surface of *B. hyodysenteriae*

has biological activity, and may contribute to lesion formation. Studies with inbred mouse strains showed that their response to LOS from *B. hyodysenteriae* was correlated with their susceptibility to infection (Nibbelink and Wannemuehler 1991). *B. hyodysenteriae* and other *Brachyspira* species contain a prophage-like gene transfer agent that may be responsible for exchange of genetic material within and between *Brachyspira* species (Stanton et al. 2008; Motro et al. 2009).

Pathogenesis

Swine dysentery can take from 5 days to several weeks to develop following experimental exposure to *B. hyodysenteriae.* Once established, the spirochete colonizes mucus deep in the crypts of Lieberkuhn within the cecum and colon. It invades goblet cells and stimulates an outpouring of mucus. Solid plugs of mucus overlie the epithelium and are present in the feces. The spirochete may invade through the junctional complex into the lamina propria, but rarely moves deep into the tissue. The main lesion is an epithelial necrosis and erosion, likely resulting from the cytotoxic action of the hemolysins. Infected pigs have increased numbers of mucosal $CD4^+$ T cells that also may contribute to lesion development through an immunopathogenic process (Hontecillas et al. 2005). The resulting eroded and bleeding surfaces become invaded by other bacteria and by the protozoan *Balantidium coli*, with the formation of extensive fibrinous plaques attached to and overlying the lesions in chronic cases. There is a failure to absorb water and electrolytes, which contributes to diarrhea. Some pigs die in the acute phase of the disease, possibly due to endotoxic shock.

Immunity

Serum antibodies and immunity develop after infection, but protection tends to be LOS serogroup-specific. Recovered pigs have an increased percentage of $CD8\alpha\alpha$ cells, which may be involved in recovery and protection from SD (Waters et al. 2000). Infected pigs also show an IgA response in the colon, but this does not necessarily correlate with protection. The spirochete has a set of genes encoding 39-kDa variable surface proteins that show differential expression, and it has been suggested that these may contribute to antigenic variation and host immune system avoidance (Witchell et al. 2006).

Brachyspira pilosicoli

Brachyspira pilosicoli colonizes the large intestine of a range of species, notably pigs, chickens, dogs, and humans. It is a cause of mild typhlocolitis and diarrhea, particularly in weaner and grower pigs, but also has been associated with wet litter and reduced egg production in adult hens. Disease caused by *B. pilosicoli* is known as “intestinal spirochetosis” or “colonic spirochetosis.”

Bacterial Virulence Factors and Pathogenesis

Few specific virulence factors have been identified. An outer membrane serine protease has been described (Muniappa and Duhamel 1997), and, as with *B. hyodysenteriae*, chemotaxis and motility are likely to be important for colonization. The spirochete apparently lacks the specific attachment and invasion determinants possessed by members of the Enterobacteriaceae (Hartland et al. 1998).

Colonization by *B. pilosicoli* can be enhanced by increasing the viscosity of the colonic contents through dietary means (Hopwood et al. 2002). The spirochete readily moves through colonic mucus, and shows a specific polar attachment to the apical membrane of the colorectal epithelium. A dense carpet of spirochetes attaches to the epithelium to form a “false brush border,” and attachment can cause displacement and loss of microvilli. The spirochete may penetrate the epithelium at the extrusion zone between crypt units, and spread extracellularly in the lamina propria. Generally there is only a minor local inflammatory response in the lamina propria. In immunosuppressed human patients, and possibly in animals, the spirochetes may establish a bacteremia. Diarrhea is thought to be due to disruption of normal colonic absorption, where large numbers of spirochetes are attached to the epithelium. In chronic cases the spirochete may no longer be found attached to the epithelium, but are present in the lumen of the crypts.

Immunity

The occurrence of long-term colonization by individual strains of *B. pilosicoli* suggests that local immunity is not effective at preventing colonization. Experimentally infected pigs show little or no specific serological response following colonization (Hampson et al. 2000).

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Detailed knowledge about the virulence factors and the pathogenesis of infections with gram-negative anaerobes has generally lagged that for many other bacterial species. However, major advances are now being made through whole genome sequencing, proteomics, and improved methods of genetic analysis. The application of genomic and proteomic technologies has created a major impetus toward a rapidly improved understanding of how these bacteria colonize and cause disease, specifically from the identification of potential virulence factors. In turn, this information is encouraging the development of methods for genetic manipulation, with the aim of determining the functional role of these putative virulence factors. In the next few years, the application of post-genomic methodology to the study of the expression and interactions of such factors *in vivo* should ensure that further rapid progress is made toward the understanding of the mechanisms of pathogenesis of the associated animal diseases. Such studies are an important prerequisite for the development of improved methods for their diagnosis, control, and treatment.

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28
Leptospira

B. Adler and A. de la Peña Moctezuma

LEPTOSPIRA—THE BASICS

Leptospires are helical bacteria, about 0.1 µm in diameter by 6–20 µm in length, within the genus *Leptospira,* family *Leptospiraceae,* order Spirochaetales (Faine et al. 1999). The genus contains pathogenic and saprophytic species, with at present 13 pathogenic *Leptospira* species: *Leptospira alexanderi, Leptospira alstonii, Leptospira borgpetersenii, Leptospira inadai, Leptospira interrogans, Leptospira fainei, Leptospira kirschneri, Leptospira licerasiae, Leptospira noguchi, Leptospira santarosai, Leptospira terpstrae, Leptospira weilii*, and *Leptospira wolffii*, comprising in turn more than 260 serovars. It is expected that additional species exist. Saprophytic species of *Leptospira* include *Leptospira biflexa, Leptospira meyeri, Leptospira kmetyi, Leptospira vanthielii, Leptospira yanagawae*, and *Leptospira wolbachii,* and contain more than 60 serovars. The serovar classification of *Leptospira* is based on the expression of the surface-exposed epitopes in a mosaic of the lipopolysaccharide (LPS) antigens, and is largely unrelated to the genetic species classification. For example, closely related serovars may belong to different species.

Leptospires have distinctive hooked ends (fig. 28.1). Two periplasmic flagella are inserted subterminally, extend toward the center of the cell, and are responsible for motility; the FlaA and FlaB proteins constitute the flagellar sheath and core, respectively. Electron microscopy showed a *flaB* mutant to be deficient in endoflagella and nonmotile (Picardeau et al. 2001).

Leptospires have a typical gram-negative cell structure, but with the peptidoglycan layer attached to the inner cytoplasmic membrane and overlaid by an outer membrane. This property has facilitated ready purification of outer membranes by simple detergent extraction (Cullen et al. 2004). Within the outer membrane, the LPS constitutes the main antigen and the principal surface component. It is structurally and immunologically similar to LPS from gram-negative organisms, but is much less toxic in most tests for endotoxic activity. For example, it is 12 times less lethal for mice when compared with *Escherichia coli* LPS (Faine et al. 1999). Leptospiral lipid A contains some unusual features (Que-Gewirth et al. 2004), including a modified glucosamine disaccharide unit that is phosphorylated and methylated. In addition to LPS, structural and functional proteins form part of the leptospiral outer membrane. A large proportion of such proteins are lipoproteins with relative abundance on the cell surface: LipL32 > LipL21 > LipL41 (Cullen et al. 2005). Integral membrane proteins such as the porin OmpL1 (Shang et al. 1995) and the type two secretion system (T2SS) secretin GspD (Rodríguez Reyes et al. 2005), are also located in the outer membrane of *Leptospira* and have been shown to be antigenic. The Lig proteins possess bacterial immunoglobulin-like domains with 90 amino acid tandem repeats. LigA and LigB recognize mammalian cell matrix molecules, thereby allowing pathogenic *Leptospira* to bind to host extracellular matrix components, such as fibronectin, fibrinogen, laminin, and collagen (Choy et al. 2007; Lin et al. 2008). The expression of Lig proteins is regulated by osmolarity (Matsunaga et al. 2007) and they have potential as possible

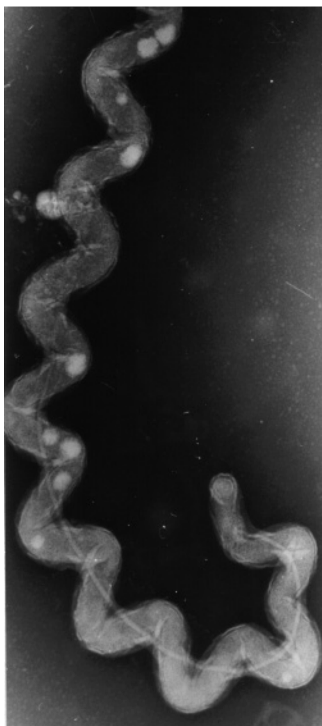

Figure 28.1. Electron micrograph of *Leptospira* sp. showing characteristic helical shape, periplasmic flagella, and outer membrane.

vaccine candidates and/or as diagnostic antigens (Palaniappan et al. 2006a).

Leptospires are obligate aerobes with an optimum growth temperature of 28–30°C. Their nutritional requirements are simple, but different from those of other eubacteria. They grow in media enriched with vitamins B1 and B12, long-chain fatty acids, and ammonium salts. Long-chain fatty acids are utilized as the sole carbon source and are metabolized by β-oxidation (Faine et al. 1999). Growth of leptospires in media containing serum or albumin and in protein-free synthetic media has been described. Several liquid media enriched with rabbit serum were described in the past by Fletcher, Korthoff, Noguchi, and Stuart (Faine et al. 1999). Currently, the most widely used medium is based on the oleic acid, bovine serum albumin, and polysorbate (Tween) medium EMJH. Some strains require the addition of pyruvate or rabbit serum for initial isolation. Growth of contaminants from clinical specimens can be inhibited by the addition of 5-fluorouracil, neomycin, nalidixic acid or rifampicin (Faine et al. 1999). Growth of leptospires is often slow on primary isolation, and cultures must be retained for at least 13 weeks before being discarded. Some very fastidious strains may require even longer incubation periods. Agar may be added at low concentrations (0.1–0.2%). In such semisolid media, growth reaches a maximum density in a discrete zone beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds. This growth is related to the optimum oxygen tension and is known as a Dinger's ring or disk. Leptospiral cultures are maintained by repeated subculture or by storage in semisolid agar containing hemoglobin. However, long-term storage in liquid nitrogen is the preferred method of storage for master stocks for vaccine production or experiments requiring maintenance of virulence.

LEPTOSPIROSIS—THE DISEASE

Leptospirosis is a systemic disease of humans and domestic animals, mainly dogs, cattle and swine, characterized by fever, renal and hepatic insufficiency, pulmonary manifestations, and reproductive failure. Clinical signs are quite variable; most cases are probably inapparent and associated with host-adapted serovars such as Canicola in dogs, Bratislava in horses and pigs, Hardjo in cattle, and Australis and Pomona in pigs (Ellis et al. 1986; Bernard 1993; André-Fontaine 2006; Grooms 2006). However, other serovars can be involved in more serious disease.

Leptospirosis in Dogs

Dogs represent a common source of infection for humans; in a study on an open population of asymptomatic dogs, 6.9% were urine-culture positive, with all isolates genetically characterized as serovar Canicola by multiple locus sequence typing (MLST; Castillo Sánchez et al. 2009). Four syndromes have been identified in canine leptospirosis: icteric, hemorrhagic, uremic (Stuttgart disease), and reproductive, (abortion and premature or weak pups). Typical leptospirosis in dogs may present with fever, jaundice, vomiting, diarrhea, intravascular disseminated coagulation, uremia caused by renal failure, hemorrhages, and death (Bolin 1996). Respiratory distress is sometimes associated with pulmonary hemorrhages seen at necropsy. In the 1980s, acute, usually fatal, hemorrhagic gastroenteritis in 3–6 month-old pups caused by *Leptospira* was commonly

misdiagnosed as parvovirosis infection (Hartman et al. 1986). Stuttgart disease, the uremic presentation of leptospirosis in dogs, includes vomition, polydipsia, oliguria, and halitosis due to the presence of oral ulcers. Less commonly seen, but not rare, are cases of abortion or delivery of weak pups that usually die during their first week.

Leptospirosis in Cattle

In cattle, signs of leptospirosis include abortion, stillbirth, and birth of premature and weak calves; infection is worldwide. In addition, infertility and early embryonic death are also associated with infection with serovar Hardjo (either subtype Hardjobovis or Hardjoprajitno). Abortion storms may occur after infections with serovars Pomona or Grippotyphosa, but sporadic abortions are more typical of serovar Hardjo infection. Abortions typically occur without premonitory signs, one or more weeks (Pomona, Grippotyphosa) or months (Hardjo), after infection of the cow. Abortion caused by serovar Pomona has decreased in importance, probably because of vaccination. Although the precise prevalence is unknown, reports vary from 10% to 50%, with most infections probably caused by serovar Hardjo. Many leptospiral infections are subclinical, particularly in nonpregnant animals, and are detected mainly by the presence of circulating antibodies. The pathogenesis is not clear, but is likely related to the persistent localization of leptospires in the uteruses and oviducts of Hardjo-infected cattle (Grooms 2006).

Leptospirosis in Pigs

Leptospirosis leads to economic losses in the pork industry worldwide; it has also been an important source of leptospiral transmission to humans (Schollum and Blackmore 1982). In pigs, leptospirosis causes abortion, stillborn and weak piglets, fetal mummification, and deaths soon after birth. In newborn piglets, subcutaneous hemorrhages, hematuria, jaundice, and death might be seen; growing pigs may be weak, lethargic, and icteric (Ellis 1999). Antibodies to leptospires of serovars Bratislava, Canicola, Icterohaemorrhagiae, Pomona, and Tarassovi have been detected in pigs worldwide. Serovar Pomona causes abortions, fetal deaths, premature farrowing, stillbirths, and the birth of weak piglets that grow poorly or do not survive; serovars Bratislava and Icterohaemorrhagiae are linked to stillbirths.

Leptospirosis in Horses

A chronic manifestation of leptospirosis is commonly seen in horses as recurrent uveitis (Rohrbach et al. 2005), but is not unique to this species and may also be seen occasionally in humans. Horses experimentally or naturally infected with *L. interrogans* developed clinical uveitis (equine recurrent uveitis [ERU]), 1–2 years after infection. One study revealed that up to 52.8% of equine vitreous humor samples with ERU were positive for *Leptospira* via culture and 71% via polymerase chain reaction (PCR; Wollanke et al. 2004). Horses with ERU also have increased antibodies to *Leptospira* in their vitreous humor compared with serum antibodies, suggesting a persistent ocular infection with *Leptospira*. Cross-reactivity among antigens of equine cornea, lens, and leptospires has been documented by Western blot. Local immune mechanisms also contribute to the pathogenesis of equine recurrent uveitis.

Leptospirosis in Wildlife

Rhinoceroses, wild carnivores, peccaries, marine mammals, opossums, deer, raccoons, platypus, and native mice among other wild species both free living and captive, have been found as reservoirs of pathogenic leptospires. Clinical cases have also been documented. A relationship with domestic and peri-domestic mammals has been established in some, but not all cases. In addition, wild fauna in captivity has been documented as a human health risk factor. *Leptospira* serovars usually include those found in the domestic animals present in the corresponding region, such as cattle, pigs, and dogs (Mitchell et al. 1966; Mikaelian et al. 1997; Mason et al. 1998; Godinez et al. 1999; Fischer-Tenhagen et al. 2000; Colagross-Schouten et al. 2002; Colegrove et al. 2005; Ayanegui-Alcerreca et al. 2007; Loewenstein et al. 2008; Millán et al. 2009; Jori et al. 2009).

Human Leptospirosis—A Worldwide Zoonosis

It is not within the limit of this chapter to cover in detail the varied clinical aspects of human leptospirosis. More details on specific clinical presentations in humans can be found in the following reviews: Faine et al. 1999; Levett 2001; Bharti et al. 2003. Leptospirosis in humans is always acquired from an animal source; human-to-human transmission is so

rare as to have no clinical relevance and the disease is regarded worldwide exclusively as a zoonosis. Pathogenic leptospires inhabit the proximal renal tubules of carrier animals, although depending on exposure, infection may also be acquired from other tissues and organs. Viable, infectious leptospires are excreted in urine, which then serves as a source of contamination of soil and surface water, as well as flowing streams and rivers. Infection of humans and other animals thus occurs either from direct contact with urine or more commonly from indirect contact with contaminated water; major outbreaks commonly occur following flooding or heavy rainfall. Common carriers are usually species of wild or domestic animals, especially rodents and small marsupials, cattle, pigs, and dogs (fig. 28.2). Leptospires have been isolated from almost every species of mammal (including aquatic and marine mammals) and marsupials worldwide. A chronic carrier state does not follow acute infection in humans, although patients may shed leptospires in their urine for some weeks.

The severity of leptospirosis in humans may vary substantially according to the dose and serovar of *Leptospira*, and the general health and immunological status of the patient. It ranges from a mild, influenza-like illness to a severe infection with renal and hepatic failure, pulmonary distress, and death (classic Weil's disease). In developed countries infection is most frequently associated with occupational exposure and therefore occurs most commonly in agricultural workers, farmers, abattoir workers, and others whose occupation or recreational activities bring them in contact with

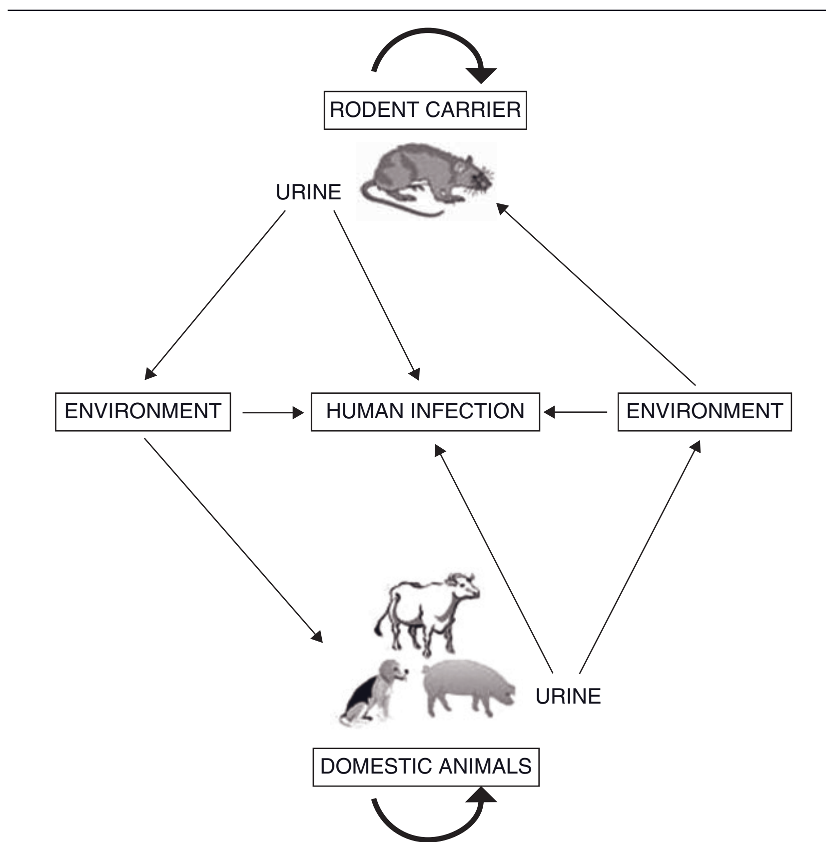

Figure 28.2. Epidemiology of *Leptospira* in rodents, domestic animals, and humans. Cycles are maintained in wild and domestic animals via infected urine and vertical transmission. Humans become infected through direct or environmental contact with infected animal tissues or urine.

carrier animals. The range of serovars is usually restricted. In tropical countries there is a universal risk from rodent-carrier mediated infection, where many serovars may be present in a locality. There are characteristic associations of particular serovars with certain species of animals as carriers (e.g., Hardjo with cattle, Canicola with dogs, Copenhageni with rats), but the association is not absolute and the molecular basis for this so-called maintenance host "specificity" is unknown.

The renal carrier state is thus the single most important factor in the persistence and epidemiology of leptospirosis. Leptospires colonize the surfaces of renal proximal tubular epithelial cells, but the molecular basis for this bacterial-cell association has not been defined. Several specific adhesion factors for host components have been described, but their roles in disease have not been elucidated. For example, the fibronectin-binding protein LigB is not necessary for virulence (Croda et al. 2008), while the major surface protein LipL32 binds host extracellular matrix (ECM; Croda et al. 2008; Hauk et al. 2008; Hoke et al. 2008), but is not required for either acute infection or kidney colonization (Murray et al. 2009c). Mechanisms of pathogenesis are discussed in more detail below. Excretion of leptospires in urine may be intermittent or continuous, with urinary concentration of bacteria as high as 10^8/ml. Leptospires do not survive well in acid urine, but can remain viable for extended periods of time in alkaline urine. Consequently, herbivores and animals whose diet produces an alkaline urine are relatively more important as shedders than are producers of acid urine.

Leptospirosis commences suddenly with acute symptoms that may include, but are not restricted to, headache, fever, malaise, myalgia, conjunctival suffusion, and sometimes a skin rash. Thereafter the illness may be mild and self-limiting or severe and fatal. The mild type may be serious and require hospitalization, but seldom leads to kidney or liver failure, hemorrhages, or death. This description is applicable worldwide to illness characteristically seen commonly in infection with serovars such as Hardjo, where fatalities are virtually nonexistent. However, infection with some of these usually milder serovars can occasionally lead to severe leptospirosis that may be life threatening. For example, severe cholecystitis has been reported for Hardjo infections. The initial symptoms may be followed by transient remission, which may then proceed to an exacerbation and include "aseptic" meningitis, renal failure, and abdominal or chest pains, reflecting the generalized pathology resulting from the multi-organ spread of leptospires. Differential diagnosis is required between leptospirosis and severe influenza, viral meningitis, and other viral infections such as yellow fever, hantavirus infection, and dengue. Complete recovery usually follows, but weakness, tiredness, depression, and psychosis may prevent a return to normal life for weeks or even many months. Reports of chronic or recurrent leptospirosis have usually not been adequately proven or investigated, but there is sufficient anecdotal evidence for these claims to be taken seriously. The cellular and molecular basis for chronic or persistent leptospirosis remains unknown. Uveitis following leptospirosis is well described in horses and so it is significant that a clear association in humans was described between uveitis following leptospiral infection and the presence of leptospires (detected by PCR) in the anterior chamber of the eye (Chu et al. 1998).

Severe leptospirosis is characteristically seen in infections with serovars Icterohaemorrhagiae, Copenhageni, Lai, Autumnalis, Pomona, and many others. The source of infection is most commonly rats or other rodents. The illness worsens, usually rapidly after onset, so that renal failure may occur within 7–10 days, sometimes accompanied or followed by skin and mucosal hemorrhages, jaundice, hemoptysis, pulmonary hemorrhages, or liver failure, leading to death if untreated. Case fatality rates approaching 20% have been reported (Levett 2001; Bharti et al. 2003). A more recently recognized respiratory manifestation involves severe pulmonary edema and hemorrhages, which have been the main cause of death in some epidemics. Indeed, mortality rates in pulmonary leptospirosis may exceed 50% (McBride et al. 2005). As with mild leptospirosis, chronic, long-term sequelae have been reported, but frequently not investigated fully.

Leptospirosis, whether mild or severe, in pregnancy carries the risks of intrauterine infection and fetal death in humans as well as in animals.

GENOMICS, PROTEOMICS, AND MOLECULAR BIOLOGY

Leptospires grow slowly in liquid culture media and, on solid media, up to 4 weeks may be required for colonies to appear. Recent clinical isolates from animals or humans frequently grow even more

poorly *in vitro* and may not form colonies on solid media at all. In addition, their relative metabolic inertness in usual phenotypic tests, such as the ability to ferment different sugars or utilize particular amino acids, has hindered classical genetic studies. In early work, mutants were selected to grow in media containing either boiled, as opposed to fresh, serum or in the presence of antiserum to the parent strain. However, the genetic basis of these mutations was never defined. Likewise, the straight (unhooked) variants that appear spontaneously as small colonies in some strains (Faine and van der Hoeden 1964) were never defined genetically.

Naturally occurring plasmids have not been detected in *Leptospira* and the mechanisms of gene transfer assumed to occur between leptospires have not been elucidated. Transformation in nature has also not been reported among leptospires. Early reports of bacteriophage-like particles associated with pathogenic *Leptospira* species were never confirmed and no phage has been found that affects any pathogen. However, a bacteriophage with a 70-kb genome, LE1, was identified in *L. biflexa*; LE1 has the unusual property that it is one of the very few phages that replicates in its temperate prophage state as a circular plasmid. This key feature formed the basis for development of a plasmid shuttle vector, pGKLep4, which replicates in *E. coli* and *L. biflexa* (Saint Girons et al. 2000). A fragment carrying the LE1 origin of replication together with the *parA* gene encoding a partitioning protein was cloned into the *E. coli* plasmid pGEM. Interestingly, the kanamycin resistance gene from *Enterococcus faecalis* was necessary for selection in *L. biflexa*. Unfortunately, it does not replicate in pathogenic *Leptospira* spp.

The genome of *Leptospira* shows a G + C content of 35–41 mol%, depending on species. The genome size varies from 3.9 to 4.6 Mb. There are currently six available published leptospiral genome sequences: two serovars (Lai and Copenhageni) of *L. interrogans*, two strains of *L. borgpetersenii* serovar Hardjo, and two strains of *L. biflexa* serovar Patoc (Ren et al. 2003; Nascimento et al. 2004; Bulach et al. 2006; Picardeau et al. 2008). The essential features of these genomes are summarized in table 28.1. The pathogenic species *L. interrogans* and *L. borgpetersenii* both have two circular chromosomes and both genomes are characterized by a high degree of genome plasticity, with evidence of large-scale genomic rearrangement mediated by the presence of insertion sequence (IS) elements. The most striking difference between *L. interrogans* and *L. borgpetersenii* is the much larger number of pseudogenes, intact ISs and IS remnants in the latter. For example, *L. borgpetersenii* contains more than 80 complete and 25 partial copies of IS*1533*, as well as 14 novel IS elements. The *L. borgpetersenii* genome is approximately 700 kb smaller and has a lower coding density than that of *L. interrogans,* suggestive of genome degradation through a process of IS-mediated reduction of the functional genome. Many genes that are intact in *L. interrogans* have accumulated disruptive point mutations IS element insertions in *L. borgpetersenii*. However, this loss of gene function is not random, but is centered on genes involved in environmental sensing and metabolite transport and utilization. In contrast, *L. interrogans* shows little evidence of genetic decay, consistent with its more robust survival in aquatic and other environments before infecting a new host. *L. borgpetersenii* survives poorly in the environment and it was therefore concluded that it is in the process of evolving toward dependence on a more direct host-to-host transmission cycle (Bulach et al. 2006).

The saprophyte *L. biflexa* also has two main circular chromosomes, but in addition possesses a

Table 28.1. Essential Features of the Genomes of Pathogenic and Saprophytic *Leptospira* spp.

	L. borgpetersenii	*L. interrogans*	*L. biflexa*
Size (kb)	3931	4627	3956
Number of genes	2844	3379	3590
Coding density (%)	80	75	92
Number of pseudogenes	368	41	33
Number of transposases	246	26	9
Number of unique genes	265	627	1348

third circular replicon of 74 kb, designated p74, not present in the pathogens. The presence of housekeeping genes on p74 that have orthologs located on the large chromosome in pathogenic *Leptospira* suggests that p74 is essential for the survival of *L. biflexa*. For example, the *recBCD* housekeeping genes are located on the large chromosome in both *L. interrogans* and *L. borgpetersenii,* but are located on p74 in *L. biflexa.*

Comparative genomics of the two pathogenic and one saprophytic species has identified 2052 common genes, the core leptospiral genome, a finding consistent with a common origin for leptospiral saprophytes and pathogens. From a pathogenesis viewpoint, genome comparisons also allow the identification of pathogen-specific genes, which might provide information about pathogenic mechanisms. The overall proportion of genes encoding proteins of unknown function is ~40% across all the leptospiral genomes. However, genes of unknown function are overrepresented in the genes unique to pathogenic species. Of the 627 genes unique to *L. interrogans*, more than 500 (80%) encode hypothetical proteins. For *L. borgpetersenii* more than 200 out of 265 (75%) unique genes encode proteins of unknown function (table 28.1; Picardeau et al. 2008). These data are consistent with the hypothesis that *Leptospira* possesses unique virulence factors that therefore cannot be identified by similarity to those of other bacteria. Pathogenic mechanisms in leptospirosis are discussed in the next section of this chapter.

The ability to construct genetically defined mutants has only recently been achieved for *Leptospira*. The first report of successful gene inactivation by recombination-mediated allelic replacement in *Leptospira* utilized the kanamycin resistance gene from pGKLep4 to inactivate the *flaB* gene, encoding the flagellar subunit protein, in *L. biflexa* (Picardeau et al. 2001). Mutants were nonmotile and lacked flagella and hooked ends, but retained their helical shape. The commonly used *sacB* gene was unsuitable as a counter-selectable marker in *L. biflexa*, but the *rpsL* gene that encodes the S12 ribosomal protein was used to increase dramatically the number of double crossover transformants obtained. Allelic replacement in pathogenic *Leptospira* spp. has proven to be more of a problem, with only one report of successful mutant construction to date. The *ligB* gene of serovar Copenhageni was disrupted with a spectinomycin resistance cassette by site-directed, homologous recombination (Croda et al. 2008); interestingly, the mutation had no effect on virulence. However, random transposon mutagenesis is now possible and the Mariner-based transposon *Himar1* has been used to construct defined mutants in several pathogenic serovars (Bourhy et al. 2005; Ristow et al. 2007; Murray et al. 2008, 2009a, 2009b, 2009c). DNA can be introduced either by electroporation or by conjugation with *E. coli* (Picardeau 2008). Identification of the disrupted gene by direct sequencing of genomic DNA has allowed the high-throughput generation of libraries of defined mutants on a scale not previously possible and has facilitated the recognition of the roles of several genes in virulence (Murray et al. 2009a).

The availability of genome sequences has facilitated investigations of the leptospiral proteome, the outer membrane subproteome, and how this varies under a range of environmental conditions. A global analysis of the outer membrane proteins (OMPs) of serovar Lai identified several novel OMPs and defined their differential regulation in response to simulated *in vivo* conditions (Cullen et al. 2002). A subset of these proteins was shown by *in situ* labeling with biotin to be exposed on the leptospiral surface (Cullen et al. 2005); the major components of the surfaceome were the lipoproteins LipL32, LipL21, and LipL41.

Like other bacterial species, *Leptospira* may change its protein profile in response to the host environment. Identification of proteins expressed *in vivo* may indicate a role in pathogenesis, but is hampered by the difficulty in obtaining sufficient amounts of bacterial proteins, free of host proteins, for analysis. Nonetheless, a proteomics study of leptospires derived from infected guinea pig liver identified LipL32, as well as several other known and novel proteins (Nally et al. 2007). Notably, the OMP Loa22 showed a marked increase in expression and was subsequently shown to be essential for virulence (Ristow et al. 2007). A later study on leptospires from the urine of carrier rats showed differential protein expression (Monahan et al. 2008), with a reduction in reactivity of antigens of urinary leptospires with immune rat serum. Again, LipL32 was identified as a prominent antigen, yet was later shown by others to be completely unnecessary for the establishment of the chronic renal carrier state in rats (Murray et al. 2009c).

Whole genome microarrays have been used to investigate the transcriptional response of *Leptospira*

to identify genes involved in the transition of leptospires from environmental temperature and osmolarity to those encountered during infection of the mammalian host (Lo et al. 2006; Matsunaga et al. 2007). The majority of genes with altered expression encoded proteins whose function was either unknown or poorly defined, again consistent with the notion that *Leptospira* produces unique virulence factors. Interestingly, genes that were up-regulated in response to physiological temperature were over represented among those up-regulated in response to physiological osmolarity. Intriguingly, the LigB lipoprotein is markedly up-regulated at physiological osmolarity (it is virtually undetectable under hypotonic *in vitro* growth conditions), yet a *ligB* mutant retained full virulence (Croda et al. 2008), consistent with a degree of redundancy in genes potentially involved in pathogenesis.

A proviso that one must bear in mind when interpreting the above range of studies by many researchers is that results from any one leptospiral species, serovar, or even strain may not necessarily be extrapolated to others. Likewise, results obtained from any animal species will not necessarily reflect the situation in other species.

PATHOGENESIS OF LEPTOSPIROSIS

The entry of leptospires into the body is similar among different animal species and occurs via mucous membranes or damaged skin. The subsequent bacteremic phase may last for up to 7 days or occasionally longer. When the numbers of leptospires in the tissues reach a critical level, lesions due to the action of undefined leptospiral toxin(s) or toxic cellular components and consequent symptoms appear. The primary lesion is damage to the vascular endothelium, particularly of small blood vessels, leading to localized ischemia and resulting in renal tubular necrosis, hepatocellular and pulmonary damage, meningitis, myositis, and placentitis. Activation of the coagulation cascade is a frequent finding in human leptospirosis, resulting in disseminated intravascular coagulation (Chierakul et al. 2008) and also occurs in acute infection of susceptible animals. Any organ can be affected in acute leptospirosis, and multi-organ failure with hemorrhages and jaundice is common in severe cases. Once circulating antibodies appear, leptospires are removed from the circulation and tissues by opsonophagocytosis. Tissue damage, even when severe, may be reversible and followed by complete repair (e.g., kidney, liver) although long-lasting damage may occur and can lead to scarring. This outcome is well recognized in the kidneys of pigs and dogs, where it may be observed macroscopically as “white spots.”

The mechanisms by which leptospires cause host tissue damage and disease are not well defined. In particular, the molecular and cellular basis for virulence remains poorly understood, due mainly to the absence, until recently, of genetic tools for the manipulation of *Leptospira*. There have been reports dating back many years of the so-called pathogenic mechanisms, but in almost all cases the specific leptospiral component responsible for the activity investigated was not identified. For example, virulent leptospires were shown to adhere to cultured renal tubular epithelial cells (Ballard et al. 1986), but the leptospiral adhesin was not identified. Virulent leptospires are killed by the antibody and complement and by neutrophils in most animal species that have been studied, except for cattle. However, they are resistant in nonimmune hosts, due to their ability to bind host Factor H, a regulator of the alternative complement pathway (Verma et al. 2006). Additionally, pathogenic, but not saprophytic or culture attenuated strains, bind the host plasma glycoprotein C4BP, a regulator of the classical complement pathway (Barbosa et al. 2008).

There is no unequivocal evidence for classical secreted exotoxins in *Leptospira*; in fact, in the kidneys of experimentally infected guinea pigs, endothelial damage was closely associated with the presence of intact leptospiral cells or their remnants (De Brito et al. 1992), suggesting the direct involvement of leptospiral cell components and/or cell bound toxins. The ability of leptospires to invade Vero cells and to induce apoptosis in macrophages was correlated with virulence (Merien et al. 1997). Significantly, both properties were lost very rapidly upon *in vitro* subculture. A 36-kDa fibronectin-binding protein was identified in a virulent strain of Icterohaemorrhagiae, but was absent in an avirulent variant and in a saprophyte strain (Merien et al. 2000); again, the variant was not genetically defined and the protein was not identified.

Leptospiral LPS resembles standard gram-negative LPS chemically and immunologically, but possesses some unusual features. The lipid A component consists of a hexa-acylated di-saccharide unit, but the usual glucosamine residues are replaced

with an α-1,6-linked disaccharide comprising 2,3-diamino-2,3-dideoxy-D-glucopyranose units. In addition, the methylation of the 1-phosphate group is unique in lipid A chemistry (Que-Gewirth et al. 2004). Leptospiral LPS is substantially less active in standard tests for endotoxin activity, such as rabbit pyrogenicity, mouse lethality, Schwartzman reaction, and B-cell mitogenicity. Whether this reduced biological activity is due to some of the unique properties of leptospiral lipid A remains unknown. The apparent activation of only TLR2, rather than the more usual TLR4, by leptospiral LPS (Werts et al. 2001) was resolved by the finding of differential recognition of TLRs in human and mouse cells. TLR2 is the predominant human receptor, while both TLR2 and TLR4 contribute to activation in mouse cells (Nahori et al. 2005). It is possible that this differential interaction may in part explain the fact that mice and rats do not normally succumb to acute infection, but rather become lifelong renal carriers of leptospires. There is no information on TLR recognition and signaling by leptospires in other animal species.

As with all infections, the host response in leptospirosis can play a role in determining the outcome of infection. In experimentally infected hamsters a pro-inflammatory cytokine response was prominent very early in infection, with the expression of anti-inflammatory cytokines following (Vernel-Pauillac and Merien 2006). Similarly, levels of IFN-γ and TNF-α correlated with the severity of infection and lung pathology in hamsters (Srikram et al. 2008). However, infection of TNF-α Receptor-deficient mice resulted in more severe renal inflammation, suggesting that TNF-α is important in the early control of infection (Athanazio et al. 2008). No difference was observed in IL-4-deficient mice. It is difficult to assess the reported possible pro-inflammatory activity of leptospiral membrane proteins (Hung et al. 2006; Yang et al. 2006) because the effects of very small amounts of LPS in the Triton X-114 preparations used cannot be completely excluded.

Hemolytic activity has been reported in several leptospiral serovars. Not surprisingly, a range of genes encoding sphingomyelinases that are either cell-associated and/or extracellular has been identified in different *Leptospira* species (Segers et al. 1992); at least seven *sphA*-like genes were detected among the pathogenic *Leptospira*, including a postulated pore-forming hemolysin SphH (Lee et al. 2000). The significance in pathogenesis of the other Sph sphingomyelinases has not been determined experimentally, but the recent availability of genome sequences from pathogenic and saprophytic *Leptospira* spp. (Ren et al. 2003; Nascimento et al. 2004; Bulach et al. 2006; Picardeau et al. 2008) coupled with the recent development of mutagenesis systems (Bourhy et al. 2005; Murray et al. 2009a) has allowed a more detailed and genetically defined investigation of cellular and molecular pathogenic mechanisms in leptospirosis. For example, the absence of any sphingomyelinase genes in the saprophyte *L. biflexa* (Picardeau et al. 2008) argues for a role in pathogenesis for these enzymes rather than in environmental scavenging of nutrients.

The first genetically defined virulence factor in *Leptospira* was the OmpA-like, surface lipoprotein Loa22 (Ristow et al. 2007). A transposon mutant of *loa22* was attenuated in both hamster and guinea pig models of acute leptospirosis. The function of Loa22 remains unknown and it is noteworthy that a Loa22 homolog is found in *L. biflexa*. Its role in virulence may thus be an indirect one, perhaps through helping to stabilize the outer membrane of the leptospiral cell. Iron is essential for the growth of both pathogenic and saprophytic *Leptospira* species, which are capable of utilizing a range of iron sources, including ferrous and ferric salts, hemoglobin, but not lactoferrin (Louvel et al. 2006). Genome analysis showed no evidence of siderophore synthesis or secretion, but identified a range of genes encoding proteins with similarity to TonB-dependent receptors, a common pathway for iron acquisition and transport in gram-negative bacteria (Bulach et al. 2006; Louvel et al. 2006; Picardeau et al. 2008). However, in the host the most abundant source of iron is heme or heme-containing proteins. Two hemin-binding proteins have been described in pathogenic *Leptospira*, including a 44-kDa protein identified as LipL41 (Asuthkar et al. 2007); however, its claimed presence in *L. biflexa* (based on antibody reactivity) is not supported by genome sequence data (Picardeau et al. 2008). Recently, the gene *hemO*, encoding a heme oxygenase enzyme was shown to be essential for *Leptospira* to scavenge iron from hemoglobin (Murray et al. 2008). Given the importance of heme as an iron *in vivo*, it is unsurprising that HemO plays a role in virulence, although it is not essential (Murray et al. 2009b). Hamsters infected

with a *hemO* mutant showed enhanced survival and reduced or absent pathology, but were kidney culture positive, consistent with the notion that while heme is the major iron source *in vivo*, some growth of leptospires in the host tissues can be mediated by other less abundant sources.

A set of six surface proteins, termed Len-ABCDEF, possesses structural and functional similarity to mammalian endostatins and were shown to bind both the complement regulator factor H and host laminin (Stevenson et al. 2007). Interestingly, all except LenA also bound fibronectin. It is therefore perhaps surprising that inactivation of either LenB or LenE had no effect on virulence for hamsters (Murray et al. 2009a). The significance and role of these activities in pathogenesis thus remain undefined.

The major surface protein LipL32 has likewise been shown to bind both host laminin (Hoke et al. 2008) and collagen and fibronectin (Hauk et al. 2008). The crystal structure of LipL32 was recently solved (Vivian et al. 2009); LipL32 showed structural, but not sequence, homology to adhesins that bind components of the extracellular matrix, consistent with experimental data showing a similar function for LipL32. LipL32 is unique to, and highly conserved across, pathogenic *Leptospira* spp. It is therefore also surprising that it is not required either for acute leptospirosis in hamsters or for colonization of rat kidneys. A LipL32 mutant exhibited normal virulence phenotype for acute and chronic leptospirosis as well as normal *in vitro* growth and survival in water (Murray et al. 2009c). The role of this enigmatic protein in pathogenesis thus remains elusive.

A similar result was reported for the surface-exposed Lig proteins; they are present only in pathogenic species and expression was lost upon laboratory subculture with a concomitant loss of virulence (Matsunaga et al. 2003). Both LigA and LigB bind fibronectin and their expression is upregulated under conditions of physiological osmolarity (Choy et al. 2007). However, inactivation of LigB did not affect virulence for hamsters nor colonization of rat kidneys (Croda et al. 2008).

The results of the above studies all indicate a high degree of redundancy in leptospiral proteins involved in adhesion, survival *in vivo*, and renal colonization, and suggest that it may be difficult to identify and define virulence factors with a single gene inactivation strategy.

MECHANISMS OF IMMUNITY IN LEPTOSPIROSIS

Immunity in leptospirosis is mediated predominantly by antibodies in humans and most animal species, including dogs, pigs, guinea pigs, and hamsters. There is a substantial body of evidence for this assertion. Immunity can be passively transferred by convalescent human or animal serum, by experimentally produced antiserum, or by appropriate monoclonal antibodies (Mabs) such as those directed against leptospiral LPS (Jost et al. 1986), which have been shown to protect guinea pigs, hamsters, dogs, and monkeys against lethal infection. The protective capacity of these sera usually correlates with their levels of agglutinating antibodies reacting with LPS. Indeed, MAbs produced against LPS are agglutinating and opsonic (Adler and Faine 1983) and in the presence of specific antibodies, leptospires are readily phagocytosed by macrophages and neutrophils, both *in vitro* and *in vivo* in most animal species. In addition, antibodies readily lyse leptospires in the presence of complement. LPS is highly antigenic, in both native form in whole or disintegrated leptospires, or in chemically purified form; immunization with purified LPS or LPS components elicits protective immunity (Jost et al. 1989; Midwinter et al. 1990; Masuzawa et al. 1996). Significantly, LPS is a major antigen recognized by human and animal convalescent sera and immunity following naturally acquired infection is usually restricted to serovars that express serologically related LPS. This has meant that in general immunity acquired following natural infection is at best serogroup-specific. Early work on immunity among serovars that did not share agglutinating antigens (Kemenes 1964; Plesko and Hlavata 1971) did not identify the responsible antigens, but intriguingly may have involved one or more of the OMPs or lipoproteins identified many years later by molecular studies.

There is at least one major exception to this rule; mechanisms of immunity in cattle are different. Animals with high levels of serum agglutinating antibodies are not protected when infected experimentally by the more natural conjunctival route. Rather, immunity is correlated with a Th1 response mediated by IFN-γ release (Naiman et al. 2001), with consequent ramifications and considerations for bovine vaccine development. Intriguingly, antibodies from these susceptible cattle can transfer

passive immunity to hamsters, highlighting the divergence of immune mechanisms in different animal species and the caution that must be exercised in extrapolating mechanisms of immunity and pathogenesis among different species of *Leptospira* and animals.

Vaccination in Humans

This chapter does not aim to cover details of human vaccination and the reader is referred to relevant reviews for more information (Bharti et al. 2003; Koizumi and Watanabe 2005; Palaniappan et al. 2007). Prevention of leptospirosis without vaccination is difficult. Measures aimed at improving occupational hygiene, such as the use of protective clothing and avoidance of splash from urine or contaminated water are useful theoretically, but hard to implement in practice because they impede work or are unacceptable to both workers and employers. For example, it is not practical for tropical village dwellers to avoid hazardous activities such as contact with dogs or pigs and other livestock, or walking or working in wet conditions, including in soil or water, such as rice paddies, contaminated by the animals' urine.

Vaccines for humans and animals have been used since the 1920s; almost all of them were prepared from whole leptospiral cells killed by a variety of methods, including heat, formalin, phenol, irradiation, etc. (Faine et al. 1999). The use of undefined, live avirulent, attenuated, or saprophytic leptospires has not gained acceptance. Many of these early preparations were too reactogenic for widespread human use. Attempts to reduce reactogenicity have included the use of protein-free media for growth of leptospires and the use of subcellular fractions, the active component of which was almost certainly LPS.

Nevertheless, human vaccines prepared from killed leptospires have been used successfully in China, following floods resulting in exposure of large populations to risk of leptospirosis (Chen 1985), Japan, and Vietnam. Generally, vaccines contain two or more locally prevalent serovars, emphasizing the necessity of sound knowledge of the local epidemiology. For example, a trivalent killed vaccine tailored to local serovars was recently developed in Cuba following the unavailability of an imported vaccine (Martínez et al. 2004). In all cases, repeated annual revaccination is recommended to retain immunity. As with natural infection, immunity is restricted to antigenically related serovars.

Vaccination of Animals

Vaccination of livestock can reduce urinary shedding and risk to human handlers, especially when accompanied by appropriate education programs, awareness, and hygiene in the community, and support from the authorities responsible for administration of human and veterinary public health. Commercial *Leptospira* vaccines are available globally for cattle, pigs, and dogs, but vaccination has proven to be only partially effective, due in part to the serovar-restricted nature of vaccine-induced immunity and the potential presence of local serovars other than those included in the vaccines. A successful vaccination program requires continued epidemiological studies to assess the incidence of different *Leptospira* serovars in a given population. Most animal vaccines developed in the past have consisted of whole cell bacterins containing leptospires inactivated by chemical means or heat (Wang et al. 2007). In the past, live attenuated vaccines have given variable results. Kenzy et al. (1961) compared a bacterin with an attenuated vaccine obtained by passage in eggs of a Pomona virulent strain, showing that such an attenuated vaccine reduced the abortion rate in cattle. Contradictory results were observed when gamma-irradiated Icterohaemorrhagiae cells were used as a vaccine in one study (Hubbert and Miller 1965). Irradiated leptospires protected guinea pigs from disease and death, but there was no protection in a similar assay done by Babudieri et al. (1973). Live avirulent leptospiral vaccines have also been used with good results when immunized pigs were challenged (Fish and Kingscote 1973); however no acceptance or authorization was obtained due to risks in handling and stability of the product.

Vaccines for Dogs

Bivalent bacterins for dogs containing serovars Canicola and Icterohaemorrhagiae have been on the market since the 1950s. Very early studies by Broom showed that hamsters could be protected with phenol-treated Canicola cultures, while Brunner and Meyer (1950) immunized dogs and hamsters with a bacterin containing Canicola and Icterohaemorrhagiae and showed only serovar-specific protection. Current canine vaccines generally, but not exclusively, contain serovars

Canicola and Icterohaemorrhagiae. Vaccines generally protect against disease and renal shedding under experimental conditions, but transmission of serovar Icterohaemorrhagiae from immunized dogs to humans has been reported. Moreover, immunized dogs may be infected with serovars other than those contained in commercial vaccines (Prescott 2008). Recent vaccines may also include serovars Grippotyphosa and Pomona in addition to the traditional vaccine strains, in response to the increasing incidence of canine infection with these serovars. Some of these are subunit vaccines containing leptospiral outer membranes that have shown to be protective in potency trials in hamsters challenged with a serovar Canicola virulent strain.

Vaccines for Cattle

A number of *Leptospira* vaccines for use in cattle have been developed; most of these have been bacterins containing organisms inactivated by various chemical means or by heat. Multivalent vaccines have been prepared using several serovars in their formulation (Copenhageni, Pomona, Hardjo, Grippotyphosa, Autumnalis, Icterohaemorrhagiae, Canicola) with satisfactory results in guinea pigs and hamsters. Bolin et al. (1989) demonstrated that pentavalent vaccines containing serovars Hardjobovis or Hardjoprajitno did not protect cattle against challenge infection, as demonstrated by shedding of organisms in urine, although their microscopic agglutination titers (MAT) were high, suggesting that efficacy of bovine vaccines could not be based on serological response alone. Most bovine and porcine vaccines contain at least serovars Hardjo and Pomona, respectively; in North America, commercial vaccines may also contain serovars Canicola, Grippotyphosa, and Icterohaemorrhagiae. Protection against Hardjo infection in cattle has been suboptimal; for this reason, cattle may be vaccinated up to every 3 months in areas of high prevalence. In a series of landmark studies resulting in a paradigm shift of understanding of immunity in cattle, Bolin and colleagues showed that the protective efficacy of killed Hardjo vaccines in cattle correlated with their ability to stimulate a Th1 response characterized by IFN-γ release (Naiman et al. 2001, 2002; Brown et al. 2003). Vaccines that failed to elicit a Th1 response did not protect against infection although they induced high serum antibody titers.

Swine Vaccines

Leptospiral vaccines for pigs usually include one or more of serovars Pomona, Grippotyphosa, Bratislava, Canicola, and Icterohaemorrhagiae. Serovar Bratislava has been reported as abortigenic for sows. Other serovars, for example, Hardjo, do not elicit significant titers in vaccinated sows. Leptospiral bacterins have often failed to induce significant levels of agglutinating antibodies despite using adjuvants. Francois et al. (1995) showed that a single vaccination of swine with a killed leptospirosis vaccine did not induce MAT titers in 96% of animals after 15 days, but titers were 100 on day 28 after a booster 15 days post-vaccination. Similarly, the intramuscular vaccination of pigs with two doses, 2 weeks apart, of a serovar Bratislava bacterin, protected pigs against renal infection when challenged with a virulent Bratislava strain 6 weeks later, but did not protect pigs after 26 weeks (Ellis et al. 1989).

Potential Protective Antigens of *Leptospira*

The first published report of protective immunity stimulated by specific, defined protein antigens (Haake et al. 1999) involved the immunization of hamsters with *E. coli* membrane fractions containing a combination of the trimeric porin OmpL1 and the lipoprotein LipL41. This preparation induced significant, but not complete, protection against homologous challenge with *L. kirschneri* serovar Grippotyphosa. No protection was observed when either antigen was administered alone. Interestingly, purified non-membrane-associated forms of the antigens were not protective, even when used in combination, leading the authors to speculate that the manner in which these proteins associate with the membrane is important in the induction of a protective immune response. Since that report, several additional OMPs have been evaluated, with varying degrees of success.

LipL32

The leptospiral outer membrane is dominated by the lipoprotein LipL32 that makes up more than 50% of both the outer membrane subproteome (Cullen et al. 2002) and the surfaceome (Cullen et al. 2004, 2005). Despite this abundance on the leptospiral surface, attempts to stimulate protective immunity with LipL32 have yielded equivocal results. For example, immunization of gerbils

with a recombinant adenovirus expressing LipL32 yielded statistically significant protection (Branger et al. 2001); however, the biological significance of this result must be tempered by the fact that 50% of control unimmunized animals also survived. A follow-up study that used *lipL32* DNA immunization (Branger et al. 2005) likewise found survival rates of 60% in immunized animals compared with 35% in controls, yielding an insignificant P value of 0.18. Similar problems arise when assessing the results of attempts to immunize hamsters with LipL32 expressed in *M. bovis* BCG (Seixas et al. 2007). A proper statistical analysis shows that significance was achieved ($P = 0.022$, Fisher's exact test) in only one of three experiments, although pooling of the total data yields significance. Two anti-LipL32 Mabs provided 100% protection against lethal infection in hamsters, but not against renal colonization (Maneewatch et al. 2008), although a control group receiving an unrelated Mab was not included in this study. Why then has it been so difficult to stimulate protection with this major surface protein? LipL32 is expressed during infection and elicits an antibody response (Flannery et al. 2001), yet immunity following natural infection is usually serogroup-restricted. Why are these antibodies apparently not protective? One can hypothesize at least two possible protective mechanisms. Firstly, antibodies may opsonize leptospires for phagocytosis. In the case of LipL32, antibodies produced during infection may be directed against epitopes that are not accessible on the leptospiral surface. Since neither the structure nor the membrane topology of LipL32 is known, this possibility remains speculative. Alternatively, antibodies may act to inhibit or neutralize some biological activity. LipL32 has clearly been shown to bind mammalian ECM proteins (Hauk et al. 2008; Hoke et al. 2008) and also to be involved in hemolysis. Indeed, anti-LipL32 Mabs could neutralize the hemolytic activity of serovar Pomona (Maneewatch et al. 2008). However, as with other biological properties, *Leptospira* appears to possess a high degree of redundancy in ECM adhesion. A *lipL32* mutant retained ECM-binding activity, virulence for hamsters and the ability to colonize rat kidney (Murray et al. 2009c).

LipL21

There is a single report claiming protection of guinea pigs by immunization with LipL21 delivered by DNA vaccination (He et al. 2008). However, despite clear demonstration of an immune response against LipL21, the protection results are unconvincing. All animals survived challenge, with conclusions drawn from differences in body weight not subjected to proper statistical analysis and from differences in pathology, which are by definition subjective and prone to sampling error. Work in the authors' laboratory has failed to demonstrate protection in either hamsters or C3H/HeJ mice immunized with LipL21.

OmpL1

After the initial promise of OmpL1 in stimulating protective immunity (Haake et al. 1999), there has been surprisingly little follow-up work on OmpL1 as a vaccine candidate. A report claiming protection with a DNA-delivered OmpL1 vaccine (Maneewatch et al. 2007) does not withstand proper statistical analysis, which would show no difference between control and vaccinated animals either in survival or postmortem culture results. Similar problems arise when assessing claims of protection with recombinant OmpL1 in guinea pigs (Dong et al. 2008), which presents no statistical analysis at all; when properly analyzed only 9 out of 19 groups show any protection. While OmpL1 may indeed play a role in immunity, it is difficult to draw any valid conclusions from these studies.

The Lig Proteins

The Lig proteins were identified as major components of the leptospiral surface that are not expressed under normal *in vitro* growth conditions (Matsunaga et al. 2003, 2005). As for LipL32, immunization studies with the Lig proteins have yielded variable results. Koizumi and Watanabe (2004) showed >90% protection of C3H/HeJ mice immunized with LigA and/or LigB, although the mouse is not a recognized animal model for acute leptospirosis. A study with LigA in hamsters claimed efficacy (Palaniappan et al. 2006b), but control animals showed 75% survival. A subsequent attempt to immunize hamsters with DNA-encoding LigA also claimed protection (Faisal et al. 2008); however, a proper statistical analysis of those data does not support the claim of enhanced survival ($P = 0.077$ to 0.467). Despite these shortcomings, recent work has shown unequivocal protection of hamsters with the C-terminal portion of LigA, but interestingly not with LigB (Silva et al. 2007). On the other hand,

another group was able to demonstrate marginal protection ($P = 0.01$ and 0.04) with the conserved, but not the variable, portion of the LigB protein in two out of three experiments (Yan et al. 2009). In the third experiment, no protection was observed. Analysis of pooled data yields highly significant protection, so it is therefore intriguing as to why immunity cannot be stimulated reproducibly. Despite the finding that immunization did not confer sterilizing immunity (Silva et al. 2007), LigA appears to constitute the most promising candidate antigen for a recombinant protein vaccine.

The shortcomings of these various vaccine studies highlight the importance of a proper virulent challenge and rigorous statistical analysis in the evaluation of vaccines.

DIAGNOSIS OF LEPTOSPIROSIS AND THE TYPING OF ISOLATES

Because of the wide diversity of clinical signs, diagnosis of leptospirosis is difficult and depends upon a variety of laboratory assays that detect leptospires, their products, or specific antibodies. Leptospires or their components may be detected in urine or tissues by culture, dark field microscopy, immuno-staining or PCR (Faine et al. 1999; Levett 2001; Bharti et al. 2003). Detection of leptospires by culture remains the unequivocal diagnosis (Wuthiekanun et al. 2007); however, it is hampered by slow growth rates of some *Leptospira* strains, especially fresh clinical isolates, and the long incubation periods before leptospires can be detected in culture. The successful isolation of *Leptospira* requires fresh tissue, blood or urine samples, prior to initiation of antibiotic treatment, usually inoculation of at least two 10-fold dilutions of tissue fluid or homogenate, and depending on the contamination level, 5-fluoruracil or other selective antimicrobial agents to inhibit contaminants (Faine et al. 1999; Levett 2001). Incubation for up to 13 weeks at 30°C with weekly examination by dark field microscopy is necessary before cultures can be discarded as negative; some very fastidious isolates may require even longer incubation periods. Culture is therefore not considered useful as a routine test for diagnosis in individual cases, but remains important for epidemiological purposes, so that knowledge of locally prevalent serovars remains current. Other alternative detection methods, such as dark field microscopy, immunofluorescence, immunohistochemistry, antigen ELISA, or immunoprecipitation, for detection of leptospires in urine, blood, or other tissues, lack sensitivity and/or specificity; they are not recommended for routine use but may be appropriate in certain circumstances, such as demonstration of leptospires in fixed tissues.

The MAT is the most widely used diagnostic test. It has the advantage of being specific for serovars, or at least serogroups, thus giving a good indication of the infecting serovar, but it cannot discriminate between antibodies resulting from infection or vaccination; this may cause particular problems in animals, for example, in screening for disease status to comply with import or export regulations. The criterion for considering a result indicative of current *Leptospira* infection is usually accepted as a high single MAT titer of ≥400 in the presence of clinical signs and appropriate history, or a fourfold rise in titer in paired serum samples (Faine et al. 1999). Both the sensitivity and specificity of the MAT are very high, but as with all serological tests, it is negative during the early acute stage of infection. However, the MAT may also present problems because of the requirement for live cultures of different *Leptospira* serovars prevalent in a particular geographical area. In order to achieve maximum reliability and standardization, it is recommended that fresh cultures of relevant serovars be obtained from a certified reference laboratory on a yearly basis. Participation in an accredited quality assurance program is mandatory.

Since the first report of the use of ELISA to detect antibodies in humans (Adler et al. 1980), many tests have been developed using a wide variety of antigen preparations and applied for diagnosis in most animal species. These are far too numerous to detail here; a quick search of the literature databases reveals more than 150 papers in this area. Antigens used have included sonicates and other fractions obtained from leptospiral cells, as well as recombinant proteins and lipoproteins such as LipL32, LipL41, LigA, OmpL1, and others. Most studies have focused on human sera, but tests have also been evaluated in cattle, dogs, and other species. There is little evidence for a clear advantage of any single antigen preparation, although antibodies against LigB seem to appear earlier than those detected by MAT or ELISA (Croda et al. 2007) and clearly warrant further evaluation. The ELISA obviates the need for maintenance of live cultures and is amenable to automation. However, sensitivity and specificity in most

cases do not match those of the MAT, and reliance on ELISA alone is not recommended. Other antibody detection methods include macroagglutination, latex agglutination, lateral flow assays, and IgM dipstick (Levett 2001; Bharti et al. 2003; McBride et al. 2005).

A similar difficulty occurs when evaluating PCR protocols for detection of leptospiral DNA in clinical material. Many have been developed since the 1990s, with currently more than 100 reports in the literature; most of them reported high sensitivity. The protocol of Merien et al. (1995) was a genus-specific assay that amplified DNA from both pathogenic and nonpathogenic serovars. On the other hand, the approach described by Gravekamp et al. (1993) and evaluated by Brown et al. (1995) requires two sets of primers in order to detect all species containing pathogens. In the more than 15 years since these early reports, assays targeting a range of genes have been described; they are too numerous to describe here. Targeted sequences have included genes encoding 16SrRNA, 23Sr RNA, LipL32, LipL21, RpoB, GyrB, OmpL1, LigAB, FlaB, and others. As is the case with the multitude of ELISA tests, it is impossible to make any meaningful comparisons between these different PCR assays, which have used differing primers, conditions, target sequences, and target tissues. Improved sensitivity has been achieved by quantitative PCR either using TaqMan probes (Slack et al. 2007) or SYBR green fluorescence (Levett et al. 2005).

A major problem in the identification and typing of leptospiral strains arises from the fact that phenotypic tests (e.g., sugar fermentations) commonly applied to other bacterial species cannot distinguish between species of *Leptospira*, let alone serogroups or serovars. The identification and characterization of leptospiral isolates have evolved substantially from the laborious and often unreliable cross-agglutination absorption protocols of the past (Faine et al. 1999), which have been replaced by more reliable and robust modern methods. Restriction fragment length polymorphism (RFLP) analysis (Herrmann et al. 1992) is able to discriminate strains at approximately serovar level, making this method useful in comparative studies. A drawback is the requirement for specialized equipment and expertise. On the other hand, 16S rRNA sequencing (Morey et al. 2006) was able to identify the then known eight pathogenic species that clustered in a pathogenic clade, and distinguish them from two clades containing the saprophytes *L. biflexa* and others or the intermediate species *L. inadai, L. fainei*, and *L. broomii*. Majed et al. (2005) developed a PCR-based method for typing of *L. interrogans*. They analyzed 44 loci for size variability due to the presence of variable-number tandem repeats (VNTRs) and found seven of them to be useful markers for discrimination of strains at the serovar level. Although not in widespread use, this method has been applied to the molecular typing and epidemiology of *Leptospira* isolates (Salaün et al. 2006; Slack et al. 2006).

More recently, MLST of a small number of housekeeping genes (usually seven or less) has been applied to characterizing strains of *Leptospira* (Ahmed et al. 2006). In particular, MLST was able to demonstrate the emergence of a single dominant and highly virulent clone of serovar Autumnalis that was the cause of a large outbreak of human leptospirosis in Thailand (Thaipadungpanit et al. 2007). MLST thus promises a more straightforward characterization method for *Leptospira* isolates that is amenable to standardization through online databases (www.leptospira.mlst.net), allowing ready access to current molecular epidemiology for almost every laboratory worldwide.

GAPS IN KNOWLEDGE AND ANTICIPATED DEVELOPMENTS

Knowledge of the basic biology of *Leptospira* and the mechanisms of pathogenesis in leptospirosis has been hampered by the lack of the standard genetic tools for mutagenesis, transformation, and complementation that have been available for other bacterial pathogens for many years. Comparative genomic analysis of pathogenic and saprophytic species suggests that *Leptospira* may possess unique virulence factors. However, with the recent development of transposon and targeted mutagenesis systems for pathogenic *Leptospira* spp., the next few years will bring major advances in the understanding of how *Leptospira* survives in the environment and spreads to mammalian hosts to cause disease.

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29
Mycoplasma

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INTRODUCTION

Mycoplasmas are unique procaryotes that lack a cell wall and have the smallest cell size (down to approximately 300 nm in diameter) and the smallest genomes (down to 580 kb) of all free-living organisms. The term mycoplasma is commonly used in the trivial sense to refer to members of the class Mollicutes. They are widely distributed in nature as parasites of mammals, birds, reptiles, fish, arthropods, and plants. Phylogenetically, the Mollicutes are related to the gram-positive bacteria with low genomic G + C mol %, namely *Clostridium*, *Lactobacillus*, *Bacillus*, and *Streptococcus* (Maniloff 1992), from which they have probably evolved by reductive evolution. Although phylogenetic studies of Mollicutes have been based mainly on 16S ribosomal RNA gene alignments, these have been confirmed using other genes (Wolf et al. 2004). Mollicutes are divided into four phylogenetic groups, the Pneumoniae, Hominis, Anaeroplasma, and Spiroplasma groups.

The extraordinary pathogenic and genetic plasticity of the Mollicutes is demonstrated by the presence of a cluster of highly pathogenic *Mycoplasma* species that infect only ruminants, the Mycoides cluster, within the Spiroplasma group, which otherwise consists of insect-transmitted plant pathogens. One member of the Mycoides cluster, *Mycoplasma mycoides* subspecies *mycoides* small colony type, is the cause of contagious bovine pleuropneumonia (CBPP), one of the most serious bacterial diseases of domestic animals (Gasparich et al. 2004). Species in the genera *Mycoplasma*, *Ureaplasma*, and *Acholeplasma* may be found in clinical specimens from animals, but most of the animal pathogens are members of the genus *Mycoplasma*. The hemotrophic pathogens formerly classified as *Haemobartonella* spp. and *Eperythrozoon* spp., and placed within the rickettsias, are now considered to be members of the genus *Mycoplasma* (Neimark et al. 2001).

CHARACTERISTICS OF THE ORGANISM

Because they lack a cell wall, mycoplasmas are highly pleomorphic, but some species have a cytoskeleton that controls their shape (Balish and Krause 2006). The small cell and genome sizes of mycoplasmas lead to fastidious growth requirements that are met by highly enriched laboratory media. Isolation of mycoplasmas from clinical specimens is therefore a long process (usually 1–2 weeks) that requires a specialized laboratory. Numerous polymerase chain reaction (PCR) assays have been developed as alternative diagnostic methods, but they are not always sensitive or species specific. Osmotic stability under physiological conditions is maintained by incorporation of cholesterol in the cell membrane (unique among the procaryotes); in all genera except *Acholeplasma*, cholesterol must be supplied preformed by adding serum to the medium. It is possible to grow some mycoplasmas in a nutrient broth without serum (Ramirez et al. 2008), but the cultures must first be adapted to this medium, and thus it is not suitable for direct isolation from hosts. In nature, mycoplasmas are obligate parasites that are well adapted to survive on moist mucosal surfaces of their vertebrate hosts.

Mycoplasmas are a diverse group, particularly with respect to their pathogenic potential, which varies widely both between species and among strains within species. Many species are commensals, while a few can cause acute mortality. Assessing the pathogenic role of mycoplasmas can be problematic. Their isolation from mucosal surfaces in disease states does not necessarily imply etiologic significance because the same species often can be isolated from the same sites in clinically normal animals. Factors such as coinfection with other agents and adverse environmental influences can play an important role in precipitating disease. While animal mycoplasmas are regarded as extracellular parasites, at least some species have the capacity to invade nonphagocytic cells.

SOURCES OF THE BACTERIUM

Mycoplasmas are relatively host specific. While infection may occur in more than one host species, mycoplasmas tend to persist in a primary host, to which disease is often confined. The distribution of mycoplasmas probably mirrors the distribution of their hosts, although some species are restricted nationally or locally as a result of the implementation of disease control policies in domestic livestock.

Infected animals are the principal sources of pathogenic mycoplasmas. A prolonged carrier state is common, with the organism persisting in its usual habitat on a mucosal surface. Some species persist in sequestrated lesions in carrier animals, while others have been found in unusual sites such as, for example, members of the *M. mycoides* cluster in the ears of goats (Cottew and Yeats 1981).

Pathogenic mycoplasmas may be transmitted in respiratory aerosols, in milk, and in reproductive tract secretions of infected animals. They may be transmitted by eggs in birds. Because they lack a cell wall, mycoplasmas are susceptible to desiccation and disinfectants and so do not survive in the environment for long periods. Some species can form biofilms which may provide some protection in the environment (McAuliffe et al. 2006). Lateral transmission is favored by close animal contact. Despite the delicate nature of the mycoplasma cell, transmission over considerable distances via wind or fomites is suspected under intensive animal husbandry conditions.

BACTERIAL VIRULENCE FACTORS

Adhesins

Primary interactions between the host and mycoplasma cells occur through cell surface adhesins produced by the mycoplasma. The adhesins have been characterized in only a limited number of mycoplasmas, and while there are homologs of the characterized adhesins in some other mycoplasmas, it is clear that different molecules and structures may be involved in adhesion in different species. The adhesin proteins, and in some mycoplasmas, the structures with which they are associate, have been identified through inhibition of cytadhesion with specific antibodies and selection of spontaneous or transposon-induced mutants deficient or reduced in their capacity to attach to cells.

Attachment Organelle

The best characterized adhesin is that of *Mycoplasma pneumoniae* which possesses a specialized tip or terminal organelle that functions in adherence and motility. Several components are required for the correct assembly and function of the major adhesins P1 and P30, with at least eight accessory proteins involved in the assembly (Krause and Balish 2004). The P1 protein clusters at the tip organelle enabling attachment between the tracheal cilia. The phylogenetically related poultry pathogen *Mycoplasma gallisepticum* also possesses a tip structure or bleb (fig. 29.1). The P1 homolog (GapA) and several accessory proteins have been identified in *M. gallisepticum* (Goh et al. 1998; Papazisi et al. 2003). *Mycoplasma gallisepticum* possesses the cytadhesin VlhA, previously referred to as pMGA (Markham et al. 1992). A homolog of this adhesin is found in *Mycoplasma imitans*, a pathogen isolated from ducks, geese and game birds (Bradbury et al. 1993; Markham et al. 1999). VlhA is also found in the phylogenetically unrelated poultry pathogen *Mycoplasma synoviae* (Noormohammadi et al. 1997), and its presence in *M. gallisepticum* and *M. imitans* is probably the result of past horizontal gene transfer. The *pvpA* gene of *M. gallisepticum* encodes an additional putative cytadhesin (Yogev et al. 1994; Boguslavsky et al. 2000), although its coding sequence is interrupted in the genome sequence of *M. gallisepticum* R_{low} (Papazisi et al. 2003).

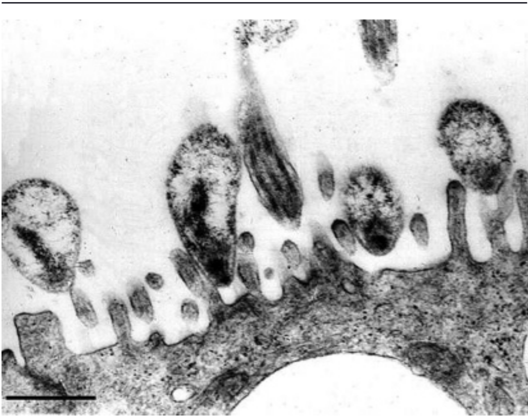

Figure 29.1. Transmission electron micrograph of *M. gallisepticum* attaching to ciliated tracheal epithelial cells via the terminal attachment organelle. Bar = 100 nm.

Ciliary Adhesin of Mycoplasma hyopneumoniae

Mycoplasma hyopneumoniae, the cause of porcine enzootic pneumonia, possesses the cell surface ciliary adhesin P97. The adhesin was identified through monoclonal antibody inhibition assays (Zhang et al. 1995), and subsequent analysis revealed two amino acid repeat sequences, R1 and R2, in the predicted sequence of the protein (Hsu et al. 1997). The R1 amino acid repeat promotes adhesion to porcine tracheal cilia (Minion et al. 2000), although the P97 paralog Mhp493, which lacks the R1 repeat, binds to tracheal cilia and heparin in a dose-dependent manner (Wilton et al. 2009). Both P97 and Mhp493 are subject to post-translational proteolytic cleavage, as is another surface protein, P159, which has also been implicated in binding to porcine cells and to heparin (Burnett et al. 2006). Thus, there appears to be a number of proteins involved in the attachment of *M. hyopneumoniae* to host cilia.

Adhesins of Other Species of Mycoplasma

The ruminant mycoplasma *Mycoplasma bovis* is an emerging pathogen in Europe, producing pneumonia, mastitis and arthritis in the host. The phylogenetically related species *Mycoplasma agalactiae*, the cause of contagious agalactia in sheep and goats, possesses a 40-kDa lipoprotein (P40) that mediates attachment to lamb synovial cells (Fleury et al. 2002). The P40 gene is present in *M. bovis*, but a frame shift mutation results in premature truncation of the protein (Thomas et al. 2004). Both *M. bovis* and *M. agalactiae* possess a family of antigenically and phase variable cell surface lipoproteins, the Vsps and Vpmas, respectively. Monoclonal antibodies against VspF and VspC inhibit attachment of *M. bovis* to bovine bronchial epithelial cells, suggesting their involvement in adherence (Thomas et al. 2003). It remains to be verified whether a 24-kDa peptide of *M. bovis* is involved in adherence to bovine bronchial epithelial cells (Thomas et al. 2005). The 150-kDa protein LppS has been identified as being involved in adherence to lamb synovial cells by *Mycoplasma conjunctivae*, a primary cause of infectious keratoconjunctivitis in domestic sheep and goats and wild Caprinae. A polyserine repeat within the carboxyl terminus of LppS may be involved in adherence, but this is yet to be confirmed (de Castro et al. 2006).

Monoclonal antibodies against the lipoproteins Maa1 and Maa2 of the rodent pathogen *Mycoplasma arthritidis* partially inhibit adherence to rat lung cells (Washburn et al. 1993). Further studies using transposon mutagenesis of *maa1* and *maa2*, followed by complementation of each with wild-type alleles, showed that Maa1 promoted adherence, while Maa2 inhibited adherence (Bird et al. 2008).

The uncultivable hemotrophic mycoplasma *Mycoplasma suis* possesses a cell surface protein MSG1 that has sequence similarity to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As GAPDH is part of the glycolytic pathway in cellular metabolism, it may play a role in the hypoglycemia observed in naturally and experimentally infected pigs. Recombinant MSG1 can bind to porcine erythrocytes in a dose-dependent manner (Hoelzle et al. 2007).

Hydrogen Peroxide

Hydrogen peroxide produced by *Mycoplasma ovipneumoniae* affects ciliary movement in ovine tracheal organ cultures (Niang et al. 1998) and is responsible for the hemolysis induced by several pathogenic mycoplasmas. Hydrogen peroxide is the final product of the flavin-terminated electron transport chain, and differences in virulence of some strains of *M. mycoides* subsp. *mycoides* small colony type have been attributed to the amount of hydrogen peroxide they produce. An ATP-binding cassette transport system for glycerol uptake is

intact in virulent African strains, while it is incomplete in less virulent European isolates; this has been proposed to account for differences in production of hydrogen peroxide, a product of the metabolism of glycerol (Pilo et al. 2005). While the levels of hydrogen peroxide produced in media by virulent *M. mycoides* subsp. *mycoides* small colony type do not affect embryonic calf nasal epithelial cells, it is suggested that the close proximity of the mycoplasma cells to the host cells allows the transfer of hydrogen peroxide across the cell membrane, producing cytotoxic effects (Bischof et al. 2008).

The levels of hydrogen peroxide produced by *M. bovis* and *M. agalactiae* have also been investigated. The repeated passage of an *M. bovis* isolate resulted in reduced hydrogen peroxide production (Khan et al. 2005). *M. gallisepticum* possesses a homolog of the *ohr* (organic hydroperoxide resistance) family (MGA_1142) that enables it to detoxify some of the reactive oxygen species found at the site of infection (Jenkins et al. 2008).

Biofilm Formation

The pathogenic mycoplasmas *M. bovis*, *M. agalactiae*, *Mycoplasma pulmonis*, and *M. mycoides* subsp. *mycoides* small colony type are all capable of forming biofilms *in vitro*, increasing their resistance to desiccation, heat, and complement mediated lysis (McAuliffe et al. 2006, 2008; Simmons and Dybvig 2007). The formation of biofilms is a two-step process, with initial binding of the organisms to a suitable substrate followed by further accumulation of organisms on these. The length of the tandem repeat region in Vsas of *M. pulmonis* is associated with biofilm formation (Simmons et al. 2007), and the Vsps of *M. bovis* may play a similar role.

Capsules

Capsules of mycoplasmas are of varying thickness and can typically be demonstrated by ruthenium red staining of the cell surface. The capsule of *M. mycoides* subsp. *mycoides* small colony type is composed of galactan and has a dramatic toxic effect in calves that are injected intravenously (Buttery et al. 1976). Their presence might be expected to aid the formation of biofilms, impede host defenses, and improve persistence in the environment.

Sialidase

The role sialidase and hyaluronidase play in the pathogenicity of *Mycoplasma alligatoris* has been investigated in alligator pulmonary fibroblast cultures (Hunt and Brown 2007). Inhibition of sialidase with 2,3-didehydro-2-deoxy-N-acetylneuraminic acid in infected cultures reduced apoptosis in a dose-dependent manner. As sialidase alone could not induce apoptosis, it has been hypothesized that sialidase acts in concert with hyaluronidase to cause apoptosis, although the presence of a virulence cofactor other than hyaluronidase cannot be ruled out. There are sialidase genes in *M. synoviae* (Vasconcelos et al. 2005) and *M. gallisepticum* (Papazisi et al. 2003), and both of these species have significant levels of sialidase activity, although its role in virulence is yet to be established (Bercic et al. 2008).

Superantigens and Lipoproteins

Superantigens elicit a massive T cell response through interaction with the major histocompatibility complex on antigen presenting cells and the T cell receptors on T cells. The arthritogenic pathogen of mice and rats, *M. arthritidis*, produces a superantigen known as MAM (*M. arthritidis* mitogen). Together with MAM of *M. arthritidis*, mycoplasma lipoproteins have been identified as nonspecific mediators of immune responses through activation of Toll-like receptor (TLR)-2 (Hasebe et al. 2007). The diacylated forms of lipoproteins appear to be potent stimulators of this response and these forms are major components of mycoplasma cell membranes.

Other Virulence Factors

The p65 antigen of *M. hyopneumoniae* is a cell surface lipoprotein with lipolytic enzymatic activity with a preference for long-chain fatty acids. In addition to its probable role in catabolism to supply nutrients, it may play a role in disease by damaging lung surfactant (Schmidt et al. 2004).

PATHOGENESIS

It is convenient to define two types of mycoplasmal disease: (1) disease resulting from invasive infections characterized by dissemination via the blood, and (2) disease where extension of infection is primarily localized. In the latter, mycoplasmemia may occasionally occur, but is not a regular feature of pathogenesis (Rosendal 1996). A list of diseases within the two categories for different hosts and their etiology is provided in tables 29.1 and 2. Where other manifestations of

Table 29.1. *Mycoplasma* Diseases of Animals Characterized by Invasive Blood-borne Infection

Primary disease	Host(s)	Species	Other manifestations
Septicemia	Goats, sheep	*M. mycoides* subsp. *capri*[a]	Polyarthritis, pneumonia, mastitis, conjunctivitis
		M. capricolum subsp. *capricolum*	Arthritis, mastitis, pneumonia
Polyserositis/arthritis	Swine	*M. hyorhinis*	Pneumonia
	Alligator (*Alligator mississippiensis*)	*M. alligatoris*	Pneumonia
Tenosynovitis/arthritis	Chickens, turkeys	*M. synoviae*	Airsacculitis
	Turkeys	*M. meleagridis*	Airsacculitis and chondrodystrophy
Arthritis/polyarthritis	Cattle	*M. bovis*	Mastitis, pneumonia
		Mycoplasma sp. bovine group 7	
		M. alkalescens	Mastitis
	Sheep, goats	*M. agalactiae*	Mastitis, conjunctivitis, pneumonia
	Swine	*M. hyosynoviae*	
	Rats	*M. arthritidis*	
	Crocodiles (*Crocodylus niloticus*)	*M. crocodyli*	Pneumonia
Hemolytic anemia	Cats	*M. haemofelis*[b]	

[a] Includes former subspecies *M. mycoides* subsp. *mycoides* large colony (LC) type and *M. mycoides* subsp. *capri*.
[b] Formerly *Haemobartonella felis*.

disease are regularly recognized, these are also included in the tables.

Invasive Infections

Invasive mycoplasmas have the capacity to penetrate epithelial barriers and enter the bloodstream, although this usually requires, or is exacerbated by, intercurrent factors. Common sequelae following a usually inapparent mycoplasmemia are localization in and inflammation of serosal cavities or joints, manifesting as one or a combination of polyserositis, tenosynovitis, or arthritis. Sometimes, more generalized infection and even acute septicemia may occur. Septicemic diseases are acute and associated with fever and often death. Infections leading to polyserositis/arthritis tend to become persistent and are accompanied by chronic inflammatory processes.

Septicemia and Multiple System Diseases

M. mycoides subsp. *capri* can cause septicemia, principally in young goats. This may occur when host immunity is poor or as a sequel of primary disease at another site (conjunctivitis, pneumonia, or mastitis). Clinical and pathological signs in kids are typical of acute septicemia (Ruffin 2001). Mortality is high, with lesions including fibrinopurulent polyarthritis, embolic pneumonia, and thromboembolic lesions in various organs indicating a generalized intravascular coagulation crisis (Rosendal 1996).

Disease associated with *Mycoplasma capricolum* subsp. *capricolum* in goats and sheep is acute and severe and typically occurs as a generalized infection, which may proceed to fatal septicemia or result in joint localization with a fibrinopurulent polyarthritis. *M. alligatoris* has also been identified as the cause of a fatal multisystemic disease, including fibrinous polyserositis, arthritis. and necrotizing pneumonia, in captive American alligators (*Alligator mississippiensis*) (Clippinger et al. 2000; Brown et al. 2001a).

Recently, a novel *Mycoplasma* sp. (*Mycoplasma zalophi* proposed sp. nov.) was isolated from several lesions in California sea lions (*Zalophus californianus*) undergoing rehabilitation. The most common

Table 29.2. *Mycoplasma* Diseases of Animals Characterized by Localized Extension of Infection

Primary disease	Host(s)	Species	Other manifestations
Pneumonia	Cattle	*M. dispar*	Mastitis, arthritis
		M. bovis	
	Sheep	*M. ovipneumoniae*	
	Swine	*M. hyopneumoniae*	
	Dogs	*M. cynos*	
	Mice, rats	*M. pulmonis*	Salpingitis, endometritis, placentitis
Pleuropneumonia	Cattle (CBPP)	*M. mycoides* subsp. *mycoides* SC type	Arthritis in calves
	Goats (CCPP)	*M. capricolum* subsp. *capripneumoniae*	
Pleuritis	Horses	*M. felis*	
Airsacculitis	Chicken/turkey	*M. gallisepticum*	Tracheitis, sinusitis, conjunctivitis
		M. synoviae	Sinusitis (turkey)
	Turkey	*M. meleagridis*	Osteodystrophy
Conjunctivitis	Cattle	*M. bovoculi*	
	Sheep, goats	*M. conjunctivae*	
	Cats	*M. felis*	
	Mice	*M. neurolyticum*	"Rolling disease"
	Songbirds	*M. gallisepticum*	
	Tortoises (*Gopherus* and *Testudo* spp.)	*M. agassizii*	Rhinitis
Vulvovaginitis	Cattle	*Ureaplasma diversum*	Infertility, abortion, pneumonia
Seminal vesiculitis	Cattle	*M. bovigenitalium*	Decreased sperm motility
Mastitis	Cattle	*M. bovis*	Pneumonia, arthritis
		M. bovigenitalium	Vulvovaginitis
		M. californicum	
		M. canadense	
		M. alkalescens	Arthritis
	Sheep, goats	*M. agalactiae, M. putrefaciens, M. capricolum* subsp. *capricolum*, and *M. mycoides* subsp. *capri*	Arthritis, septicemia, keratoconjunctivitis
		M. capricolum subsp. *capricolum*	As for *M. agalactiae*
		M. mycoides subsp. *capri*	As for *M. agalactiae*
	Goats	*M. putrefaciens*	
Erythrodermatitis	Freshwater fish (*Tinca tinca*)	*M. mobile*	Necrotizing gill lesions
Chondrodystrophy	Turkeys	*M. meleagridis*, *M. iowae*, *M. gallisepticum*	Airsacculitis, tenosynovitis/arthritis
Otitis	Cattle	*M. bovis*	Pneumonia, arthritis

SC—small colony.

lesion was subdermal abscessation, although intramuscular abscesses, septic arthritis, and lymphadenopathy were also seen (Haulena et al. 2006).

Polyserositis/Arthritis/Synovitis Syndromes

In pigs, *Mycoplasma hyorhinis* colonizes the upper respiratory tract and *Mycoplasma hyosynoviae* the tonsil without signs of illness, although *M. hyorhinis* is a common secondary opportunistic pathogen in preexisting pneumonia. However, in young pigs around weaning age, *M. hyorhinis* may, on occasion, cross the epithelial barrier and disseminate to cause acute inflammation in serosal and synovial cavities that subsequently becomes chronic. Affected pigs fail to thrive and may become runts. Serofibrinous pleuritis, pericarditis, and peritonitis are present, and in chronic cases, fibrous adhesions are prominent. Affected joints initially contain serosanguineous synovial fluid, and the synovial membranes in the chronic stages show nonsuppurative proliferative changes. Virulent strains of *M. hyosynoviae* cause arthritis in older growing pigs following stresses such as movement or vaccination. Joint lesions are similar but milder than those caused by *M. hyorhinis*. Heavy breeds are more susceptible.

Two avian mycoplasmas, *M. synoviae* and *Mycoplasma meleagridis*, are known to cause tenosynovitis. *M. synoviae* infects the respiratory tract of chickens and turkeys, usually as a subclinical infection. Strains vary greatly in virulence and some may have a tropism for synovial tissues (Kleven et al. 1975), causing clinical signs of tenosynovitis and arthritis. Exudate that is initially clear, then turbid, and later caseous is a prominent feature. Some strains of *M. synoviae* induce amyloid arthropathy, with accumulation of orange-colored exudates within joints, especially during the chronic stages (Landman and Feberwee 2001). *M. meleagridis*, a specific pathogen of turkeys, primarily causes airsacculitis and chondrodystrophy, but may be distributed in a wide range of tissues including joints, in association with synovitis (A. H. Noormohammadi, unpublished; Saif et al. 1970; Yamamoto and Ghazikhanian 1997), and bones, in association with cellular reactions (Lam et al. 2004). This suggests that this organism is invasive.

Several *Mycoplasma* species are associated with arthritis and polyarthritis in cattle (table 29.2), particularly in young cattle soon after arrival at a feedlot. With *M. bovis*, arthritis often follows a primary pneumonia and also occurs at high incidence in calves suckling cows with *M. bovis* mastitis. *M. agalactiae* is invasive and may localize in joints of sheep and goats following a primary mastitis (contagious agalactia).

Polyarthritis and subacute pneumonia caused by *Mycoplasma crocodylus* (Kirchhoff et al. 1997) has been described in farmed crocodiles (*Crocodylus niloticus*) in Zimbabwe (Mohan et al. 1995).

Chondrodystrophy in Birds

M. meleagridis is known to cause chondrodystrophic lesions, manifested as short, bowed and/or rotated bones, and deviated toes in turkey poults (fig. 29.2). These manifestations are part of turkey syndrome 65, in which poor feathering, airsacculitis, and stunting are also prominent features. Inoculation of the allantoic cavity of turkey embryos with *M. meleagridis* has been shown to induce cellular changes in association with the organism, followed by abnormal bone formation (Lam et al. 2004). This suggests that invasion of the organism into the embryonic bones may also occur following egg transmission in natural infections. The exact pathogenesis of the chondrodystrophy caused by *M. meleagridis* is unknown, although it has been speculated that the organism may deprive the growing cartilage and bone of key nutrients, in particular biotin, required for normal skeletal development. Other *Mycoplasma* species, including *Mycoplasma iowae* and *M. gallisepticum* are also reported to cause chondrodystrophy in birds (Wise 1975).

Hemotrophic Infections

The hemotrophic mycoplasmas (previously *Haemobartonella* and *Eperythrozoon* species) are predominantly epicellular parasites of red blood cells. A number of species have been described, with each specific for a particular host. *Mycoplasma haemofelis* in cats, *Mycoplasma ovis* in lambs, and *M. suis* in pigs are regularly associated with clinically apparent hemolytic anemia that is thought to result from increased erythrophagocytosis rather than intravascular hemolysis. Persistently infected animals act as infectious reservoirs and transmission occurs via transfer of infected blood, which for some species involves biting arthropods. Vertical transmission may also occur. Recently, a strain of *M. suis* has been shown to invade erythrocytes and to cause severe anemia and mortality in pigs (Groebel et al. 2009).

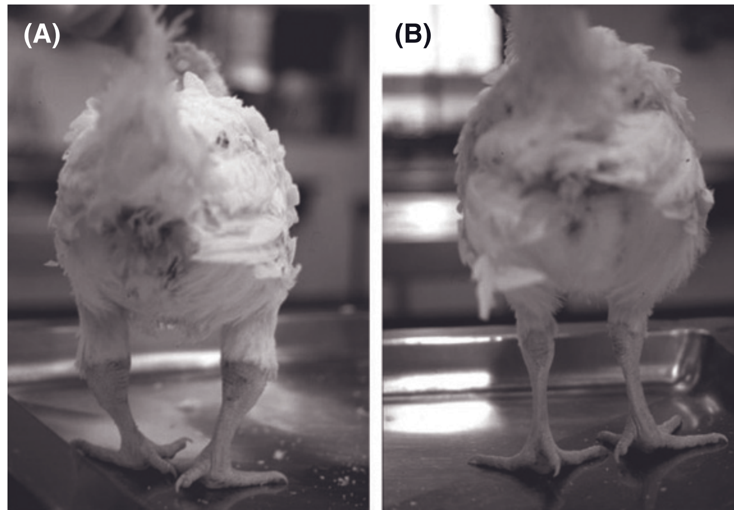

Figure 29.2. Chondrodystrophic lesions in (A) a turkey poult infected with *M. meleagridis* compared with (B) a normal turkey poult. Note the short and bowed tarsometatarsus and enlargement of the hock joint in the affected bird.

Localized Infections

Respiratory Tract Infections

There are two main manifestations of mycoplasmal respiratory disease in mammalian species. One involves bronchitis, bronchiolitis, and pneumonia and occurs in calves, lambs, pigs, dogs, mice, and rats, frequently as part of a respiratory disease complex (referred to as enzootic pneumonia in food-producing animals). The second is pleuropneumonia, with two specific well-recognized conditions: CBPP and contagious caprine pleuropneumonia (CCPP). Pleuritis in the horse is occasionally associated with *Mycoplasma felis*. In avian species, mycoplasmal respiratory disease occurs mainly as sinusitis, tracheitis, and airsacculitis.

Pneumonia. A persistent airway infection is a feature of many mycoplasmal pneumonias. The species most commonly involved are listed in table 29.2. Early stages involve direct damage by the organism to the ciliated epithelium of the bronchi and bronchioles, provoking a predominantly neutrophil and mononuclear cellular response (Rosendal 1996). This progresses to a chronic interstitial pneumonia, with lymphocytes, plasma cells, and macrophages as the predominant cell types. A characteristic of most chronic mycoplasmal pneumonias is proliferation of bronchiolar lymphoid tissue (cuffing pneumonia). Bronchiectasis is a feature of the disease caused by *M. pulmonis* in rats and mice. *Mycoplasma cynos* in dogs causes a mild bronchointerstitial pneumonia and may contribute to the kennel cough complex. *M. bovis* is more aggressive than other *Mycoplasma* species associated with pneumonia in calves, and causes lesions that include foci of necrosis. *M. hyopneumoniae* is of global economic importance as the cause of porcine enzootic pneumonia and as a key component in the porcine respiratory disease complex (Thacker 2001).

Pneumonic lesions typically occur in ventral parts of the apical and cardiac lobes of the lung, a distribution consistent with gravitational effects on the deposition of infectious aerosols and drainage of inflammatory products. It should be emphasized that pneumonic lesions caused by *Mycoplama* species alone are often mild and, in the case of *M. ovipneumoniae* (nonprogressive pneumonia) in lambs and *Mycoplasma dispar* in calves, typically subclinical. However, they may predispose to secondary bacterial infections and more severe pneumonic lesions. Complex respiratory diseases with mycoplasma as a key primary component are common and important and will be described in more detail below.

Pleuropneumonia. CBPP and CCCP are caused by *M. mycoides* subsp. *mycoides* small colony type and *M. capricolum* subsp. *capripneumoniae*, respectively. Both diseases are characterized by fibrinonecrotic pneumonia, serofibrinous pleuritis, and accumulations of serosanguinous fluid. Lung lesions may become sequestrated in a fibrous capsule and may harbor viable organisms for long periods. Animals with sequestrated lesions may be long-term carriers. Many cases of CBPP are subclinical and mortality is variable (up to 50% of diseased animals), depending on the susceptibility of the animal and the virulence of the strain involved. Strains isolated in more recent years in Europe are less virulent than classical strains. CCCP is highly contagious and mortality may reach 80%.

Respiratory Infections in Birds. *M. gallisepticum* is a parasite of the airways of chickens and turkeys and several other species of birds. Uncontrolled proliferation of the organism in susceptible birds causes severe inflammation of the mucosa of the sinuses and/or trachea, and infection frequently extends to the lungs and air sacs. Clinical signs may include tracheal râles, nasal discharge, and coughing. Gross swelling of the paranasal sinuses and ocular discharge are common in turkeys. Production may be compromised, with reduced feed consumption, egg production, and weight gain. A similar respiratory syndrome may be seen in chickens and turkeys infected with *M. synoviae*, especially when there is concurrent infection with other respiratory pathogens. However, infection with *M. synoviae* is often subclinical.

M. meleagridis infection is confined to turkeys, causing mild airsacculitis in turkey poults and being incriminated in chondrodystrophic conditions. All of the pathogenic avian mycoplasmas can be transmitted horizontally via infectious aerosols or vertically via the egg. *M. meleagridis* can also be transmitted venereally. Lesions induced by pathogenic avian mycoplasmas are mainly found in the nasal cavity, infraorbital sinuses, trachea, and air sacs, and include thickening of the mucosa, hyperplasia and/or metaplasia of the epithelial layer, infiltration and proliferation of lymphocytic cells, and infiltration of other mononuclear inflammatory cells into the lamina propria (fig. 29.3). Granulomatous changes in the lungs are also common.

M. imitans is closely related to *M. gallisepticum* and has been isolated from ducks, geese, and partridges (Ganapathy and Bradbury 1998). Under experimental conditions, *M. imitans* is not pathogenic for chickens except in mixed infections (Ganapathy and Bradbury 1999), but can cause respiratory disease similar to, but milder than, that of *M. gallisepticum*, in red-legged partridges (Ganapathy and Bradbury 1998).

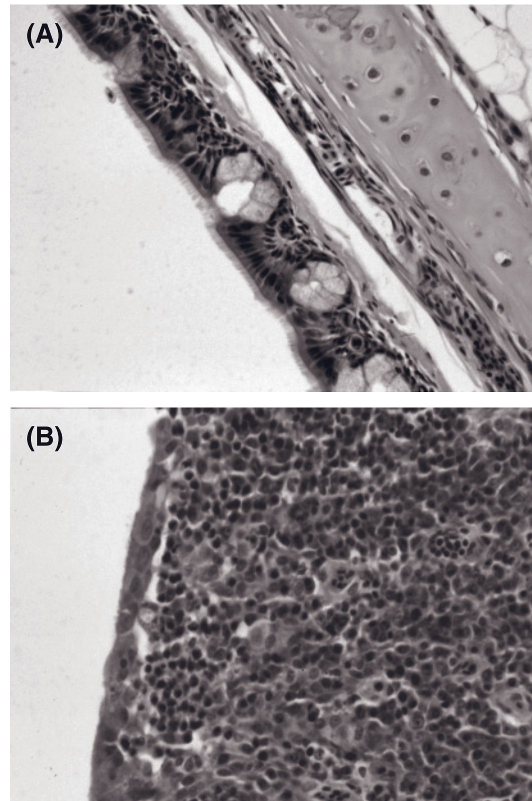

Figure 29.3. Sections of tracheas of 10-week-old chickens (A) uninfected and (B) infected 2 weeks previously with virulent *M. gallisepticum*. Panel (A) shows normal ciliated epithelium. In panel (B), there is prominent thickening caused by infiltration of the lamina propria with macrophages, lymphocytes, plasma cells, and some heterophils. Metaplasia and loss of the cilia of the epithelial cells are also evident.

Conjunctivitis. Mycoplasmas are associated with conjunctivitis in several animal species, either as a primary infection or in conjunction with respiratory disease. *M. gallisepticum* in chickens and turkeys is a good example of the latter. Conjunctivitis seems

to be a feature of recent outbreaks of disease in songbirds in North America caused by *M. gallisepticum* (Fischer et al. 1997; Williams et al. 2002). In cattle, *Mycoplasma bovoculi* causes mild conjunctivitis but predisposes to more severe infection with *Moraxella bovis*. *M. conjunctivae* causes transient conjunctivitis and keratitis in sheep, goats, ibex, and chamois, but the transient disease affects vision and can result in death through misadventure, particularly in wild chamois and ibex living in alpine environments (Giacometti et al. 2002).

M. felis is reported as a cause of conjunctivitis in cats, but it can also be isolated from the eyes of clinically normal cats. *Mycoplasma neurolyticum* occurs in the conjunctivae of clinically normal and diseased mice and is of interest because it appears to be the only mycoplasma that produces an exotoxin. When inoculated intravenously into mice, it elicits a neurological syndrome and rapid death. The syndrome is termed "rolling disease" because the mice roll around their long axis. *Mycoplasma agassizii* is widespread in tortoise populations in North America and Europe and is associated with conjunctivitis and a chronic upper respiratory tract disease (Brown et al. 2001b; Brown 2002).

Reproductive Tract Disease

Reproductive tract disease caused by mycoplasmas is relatively uncommon in animals despite their frequent isolation from this site. In chickens, colonization of the oviduct by pathogenic mycoplasmas is important in determining vertical transmission of the organism to progeny, but disease of the reproductive tract of the hen is not a feature of this infection. Some strains of *M. synoviae* can induce abnormalities of the egg shell around the apex, leading to economic loss in layer farms due to shell cracks and breaks (Feberwee et al. 2009). Granular vulovaginitis has been described in cattle (*Ureaplasma diversum* and *Mycoplasma bovigenitalium*), sheep (*M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri*), and goats (*M. agalactiae*). Prominent granules composed of hyperplastic lymphoid tissue are present on the mucosa. *U. diversum* is also an occasional cause of infertility and abortion in cows.

In the bull, *M. bovigenitalium* is a commensal of the lower urethra and prepuce. It is an occasional cause of seminal vesiculitis and epididymitis, characterized by chronic fibrosing inflammation. In such cases, it is persistently shed in the semen and seems to be associated with poor sperm motility.

Mastitis

Several species of mycoplasma are reported to cause bovine mastitis (table 29.2), but *M. bovis* is the most common and causes the most severe disease. The mammary gland in acute *M. bovis* mastitis is swollen and milk secretion is scant, with fibrin deposits and a clear whey. A persistent carrier state is common and may result in recurring clinical disease in subsequent lactations, and act as a source of infection for other cows.

Contagious agalactia is a syndrome in lactating sheep and goats caused by *M. agalactiae*. It is primarily a severe mastitis, but the organism is invasive and enters the bloodstream relatively frequently to cause arthritis, pneumonia, and septicemia. It is also associated with keratoconjunctivitis.

While *M. agalactiae* is considered the classical cause of contagious agalactia, *Mycoplasma putrefaciens*, *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri* can also cause a disease with very similar signs and lesions in goats (OIE 2004).

Gill Erythrodermatitis in Fish

Mycoplasma mobile has been isolated from freshwater fish with gill erythrodermatitis, and the disease has been reproduced with the organism (Brown 2002).

Otitis

M. bovis causes exudative otitis media in calves (Walz et al. 1997; Maeda et al. 2003). Clinical findings include unilateral or bilateral ear droop, epiphora, head tilt, and recumbency in severely affected calves. Lesions include fibrinosuppurative to caseous exudates in the tympanic bullae, ulceration and thickening of the tympanic mucosa, and infiltration and proliferation of mononuclear cells and neutrophils in the fibrous connective tissue.

INTERACTIONS

Attachment and Motility

Attachment and motility are interrelated and complementary aspects of virulence. In many bacterial pathogens, distinct, dedicated organelles, such as flagella (motility) and fimbriae (adhesion) are involved, but these structures do not exist in mycoplasmas. Rather, alternative adhesion and motility systems have evolved in these organisms. A specific

attachment organelle is found in several mycoplasmas, including the avian pathogens *M. gallisepticum* and *M. imitans*. This unique, electron-dense polar structure located at the tip of the cell controls both adherence and motility (Hatchel and Balish 2008). However, mycoplasmas that do not possess this organelle can still move and adhere to surfaces. Adhesion to host tissues relies on surface proteins that are either associated with the attachment organelle or diffusely distributed over the cell surface. Many adhesins are anchored to the cell membrane as lipoproteins, but alternate systems also exist. For example, *M. hyopneumoniae* adheres to the tips of the cilia of respiratory epithelial cells (Blanchard et al. 1992), but does not have an attachment organelle and the adhesins used are not lipoproteins (Djordjevic et al. 2004; Burnett et al. 2006).

Adhesion has been best studied in the respiratory pathogens and plays an important role in some specific virulence mechanisms. In *M. mycoides* subsp. *mycoides* small colony type, adhesion appears to be a prerequisite for the effective delivery of cytotoxic compounds such as hydrogen peroxide into host cells (Bischof et al. 2008).

Adhesion is also seen in the hemotrophic mycoplasmas, which are often found in close contact with the surface of red blood cells. In the hemotrophic porcine pathogen *M. suis*, adhesion to erythrocytes appears to be mediated by two membrane proteins, MSG1 and HspA1 (Hoelzle et al. 2007; Hoelzle 2008). Both these proteins have alternative functions in the cytoplasm, MSG1 is a GAPDH and HspA1 is a heat-shock protein. It is possible that these proteins have evolved dual functions in response to the reduction in genome size in mycoplasmas.

Another aspect of adhesion is binding to extracellular matrix components. For example, the *M. gallisepticum* surface protein MG1142, a homolog of osmotically induced proteins in other bacteria (OsmC), binds strongly to heparin and glycosaminoglycans (Jenkins et al. 2007).

The presence of multiple adhesins in the most studied pathogens suggests that adherence may be a multistep process. It is probable that initial attachment is effected by one adhesin, with subsequent closer adherence, or adherence to a different site, promoted by a second adhesin. In *M. gallisepticum*, initial attachment to the surface of the respiratory tract may be promoted by the diffusely distributed VlhA hemagglutinin. Likewise, in *M. bovis*, attachment to bovine bronchial epithelial cells is mediated by the Vsp lipoproteins (Thomas et al. 2003).

At least 12 mycoplasma species (*M. mobile*, *M. pulmonis*, *M. agassizii*, *Mycoplasma testudineum*, *M. pneumoniae*, *Mycoplasma testudinis*, *Mycoplasma amphoriforme*, *Mycoplasma pirum*, *M. gallisepticum*, *M. imitans*, *Mycoplasma genitalium*, and *Mycoplasma penetrans*) show gliding motility (Miyata 2008). This motility relies on membrane-associated proteins which are hypothesized to pass through successive cycles of attachment to a surface, movement, detachment, and repositioning. Although mycoplasmas are highly pleomorphic bacteria, such motility requires a degree of cellular organization so that the movement proceeds in the direction of the terminal organelle or an equivalent polar point on the cell (Radestock and Bredt 1977; Miyata et al. 2000). In *M. genitalium*, one protein (MG_217) appears to control the direction of cell movement by modifying the curvature of the terminal organelle (Burgos et al. 2008). Motility is likely to facilitate penetration of mucus layers and insertion of the attachment organelle between the cilia. In respiratory infections, the organisms adhere with the terminal organelle closely associated with the membranes of tracheal epithelial cells at the base of the cilia (fig. 29.1).

Ciliostasis

Within 48 h of attachment to respiratory epithelia, mycoplasmas induce ciliostasis (Power and Jordan 1976; DeBey and Ross 1994). Close attachment to the epithelial membrane is essential for this effect, implicating production of reactive oxygen intermediates and subsequent oxidative damage to the epithelial cells and their membranes, or alternatively utilization of crucial nutrients, such as arginine (Niang et al. 1998). In a process that may be involved in ciliostasis, attachment of virulent *M. hyopneumoniae* rapidly increases intracellular calcium ion concentrations in tracheal epithelial cells, acting through a cytoplasmic pathway that involves phospholipase C (Park et al. 2002). Ciliostasis reduces the efficacy of respiratory clearance mechanisms and predisposes the respiratory tract to infection with other pathogens.

Antigenic Variation

A common feature of many pathogenic mycoplasmas is the capacity for antigenic and phase variation of cell surface proteins at high frequency. The

proteins involved differ between the species, as do the mechanisms used to generate variation, but in the most dramatic example over 500,000 variants of the same protein could be generated. It is probable that in some pathogens this variation facilitates immune evasion, and in others it may enable expression of alternative functions, such as adherence to different tissues or cell types.

Vlha in Mycoplasma gallisepticum *and in* Mycoplasma synoviae

Mycoplasma gallisepticum possesses between 30 and 70 variant *vlhA* genes, most of which are translationally competent (Baseggio et al. 1996). In *M. gallisepticum* strain R_{low}, there are 43 *vlhA* genes organized into five clusters (Papazisi et al. 2003). In contrast, in *M. synoviae*, there is a large number of pseudogenes covering different discrete extents of the *vlhA* gene, organized in a single cluster (Vasconcelos et al. 2005). The *vlhA* genes have probably been acquired by *M. gallisepticum* by lateral gene transfer between these two avian pathogens. In *M. gallisepticum*, only one gene appears to be transcribed at a time, and hence only a single variant of the lipoprotein hemagglutinin VlhA is expressed on the cell surface. In *M. synoviae*, although the structural gene for VlhA is clearly homologous to those in *M. gallisepticum*, antigenic variation is achieved by a distinctly different means. There is only a single full-length gene, but there are many partial copies with variable lengths of the region of the gene encoding the carboxyl end of the protein. These partial copies can recombine at two of five specific sites in the expressed gene to generate novel, chimeric variants of the expressed gene (Noormohammadi et al. 2000). It is predicted that this has the potential to generate over 500,000 variants, with most variation generated in the region of the gene that encodes the hemagglutinin. Such variation would enable evasion of the immune response of the chicken during infection.

The control of VlhA expression in *M. gallisepticum* rests in a trinucleotide repeat region adjacent to the promoter, with genes only transcribed when they are preceded by 12 repeats of GAA (Glew et al. 1998). Switching of expression between different members of the gene family is promoted *in vitro* by antibody against the expressed gene product (Markham et al. 1998b). During infections in chickens, expression of VlhA ceases in the majority of the organisms in the respiratory tract within the first week after infection, suggesting that some signal other than antibody is responsible for this phase variation (Glew et al. 2000a). From 2 weeks after infection, an increasing proportion of the population is capable of expressing a different member of the gene family. This suggests that this adhesin may be used only in the early stages of adherence, that phase variation in expression reduces stimulation of the immune response, and expression of alternate genes ensures that antigenic variants that are not recognized by the immune response facilitate adhesion to new target cells in the chronic stages of infection.

Vlp in Mycoplasma hyorhinis

The *vlp* gene family encodes variant cell surface lipoproteins that are the immunodominant antigens of *M. hyorhinis*. Expression of the seven or more members of the family is controlled by variation in the length of a polyadenosine tract between the −10 and −35 boxes of their promoters, with genes only transcribed when the length of the polyadenosine tract is exactly 17 (Yogev et al. 1991; Citti and Wise 1995). Multiple members of the family appear to be transcribed and translated concurrently, suggesting that the purpose of this phase variation is not likely to be immune evasion.

Vsp in Mycoplasma bovis*, Vpma in* Mycoplasma agalactiae*, and Vsa in* Mycoplasma pulmonis

Mycoplasma pulmonis contains a family of at least 11 *vsa* genes encoding variable surface antigens. Only one member possesses the promoter and the amino terminal end of the coding sequence, and thus only this gene is expressed. Site-specific inversions within the locus bring this single-copy expression region into apposition with different family members, which drives variation in *vsa* transcription (Bhugra et al. 1995).

M. bovis contains a similar gene family of at least 13 genes encoding variants of the immunodominant lipoprotein Vsp (Behrens et al. 1994; Lysnyansky et al. 1999), and in *M. agalactiae* there is a family of homologous *vpma* genes (Glew et al. 2000b). Transcription of different family members is achieved by the site-specific inversion of segments of the region encoding these genes, resulting in the apposition of one gene with the single promoter sequence (Lysnyansky et al. 1999, 2001).

A site-specific recombinase that is homologous to the Xer recombinase of phage lambda is encoded by a gene adjacent to the *vpma* locus and controls the DNA inversions (Glew et al. 2002). Inactivation of this gene results in “phase locked” mutants that can only express one of the Vpma proteins (Chopra-Dewasthaly et al. 2008). Similar recombinases are suspected to play a similar role in *M. bovis* and *M. pulmonis*.

As the mechanism employed to generate variation in these gene families results in expression of a single member at a time, it is possible that it may be involved in immune evasion. When a *M. pulmonis* clone is inoculated into mice with a functional immune system, Vsa phase variation occurs at high frequency, whereas little Vsa variation occurs following infection of mice without B or T cells (Denison et al. 2005).

Vmm and Vmc in the Mycoides *Cluster*

Mycoplasma mycoides subsp. *mycoides* small colony type (strain PG1) contains a family of six *vmm* genes. Transcription is switched on or off by dinucleotide insertions or deletions in a repetitive region in the promoter (Persson et al. 2002). The six *vmm* genes are expressed *in vitro* and *in vivo*, and at least two genes can undergo high-frequency phase variation (Hamsten et al. 2008).

Another family of six variable lipoproteins, *vmc*A–F, has been found in *M. capricolum* subsp. *capricolum*. As in the *vmm* system, *vmc* phase variation is controlled by insertion and deletion of dinucleotide repeats within the promoters leading to combinatorial expression of the corresponding lipoproteins.

Size Variation of Lipoproteins

Many of the variably expressed lipoprotein genes can also vary dramatically in length. This is achieved by variation in the number of repetitions of specific short coding regions in the gene. There may be multiple different repeated regions in any one gene and different types of repeats in different members of the family. Such size variation occurs in the *vlp* family of *M. hyorhinis* (Citti et al. 2000), the *vsa* family of *M. pulmonis* (Rocha and Blanchard 2002), and the *vsp* family of *M. bovis* (Behrens et al. 1994). While the function of many of these repeated peptide sequences is only partially understood, it appears that they can shield the mycoplasma cells from complement and modulate biofilm formation (Simmons et al. 2007). In *M. hyorhinis*, longer variants of Vlp increase resistance to growth inhibitory antibody (Citti et al. 1997). In *M. pulmonis*, the expression of diverse Vsa proteins with high numbers of tandem repeats confers protection against killing by complement and promotes formation of microcolonies, whereas Vsa proteins containing fewer repeats promote cytadherence (Simmons and Dybvig 2003; Simmons et al. 2004).

Phase Variation

A number of lipoproteins of different mycoplasmas encoded by single-copy genes are also subject to high-frequency phase variation in expression. This has been observed in the MAA2 gene of *M. arthritidis* (Washburn et al. 1998), the *pvp*A gene of *M. gallisepticum* (Boguslavsky et al. 2000), and in lipoprotein genes of several human mycoplasmas. The control of expression is, in most cases, achieved by variation in the length of a repeated sequence, either in the promoter, as for the different *vlp* genes, or at the 5′ end of the coding sequence, with frame shifts resulting in premature termination of translation. In the human pathogen *Mycoplasma hominis*, this variation results in differences in adherence (Zhang and Wise 1997), but in most other cases the biological significance of this phase variation is not understood.

Antigenic Masking

A potential function of phase variation of lipoproteins may be to control access of antibody, or possibly other proteins, to constitutively expressed surface proteins. This has been best demonstrated in the human pathogen *M. hominis*, where the phase variably expressed P120 masks a constitutively expressed membrane protein, P56, from antibody (Zhang and Wise 2001). Thus, the immune evasion function of variably expressed lipoproteins may extend to protection of other cell surface proteins.

Intracellular Invasion and Fusion with Host Cells

Although most mycoplasmas are considered extracellular pathogens, several species invade eukaryotic cells *in vitro*. Invasion by *M. gallisepticum* appears to be dependent on microtubular, but not microfilament, function in the host cell (Winner et al. 2000). This species is also able to invade erythrocytes *in vivo* during infection, which could assist

colonization and dissemination in the host (Vogl et al. 2008). While persistence in the invaded cell for at least 48 h has been demonstrated, it is not certain whether intracellular replication can occur. If prolonged intracellular survival and/or replication occurs, this may help account for the chronicity of mycoplasmosis. Even in the absence of prolonged survival, intracellular invasion may facilitate penetration of the mucosal epithelial barrier.

The presence of a single bounding membrane in mycoplasmas permits direct interactions between their cell membrane and that of eukaryotic cells. Some species have been shown to fuse with eukaryotic cells *in vitro*, thus delivering their cell contents into the cytoplasm of the host cell (Rottem 2002).

A consequence of both intracellular invasion and cellular fusion is that antigens of mycoplasmas may be presented via the MHC class I molecules, thus inciting cytotoxic immunologic responses.

Lymphoid Responses

A major feature of mycoplasma infections is intense lymphoid proliferation. Studies with *M. gallisepticum* have shown that the lymphocytes infiltrating the mucosa of the trachea in the first week after infection are likely to be (NK) cells ($CD8^+$ TCR^-) (Gaunson 2000; Gaunson et al. 2000). In the second and third weeks after infection, there is an influx of helper T cells ($CD4^+$ TCR^+) and an increasing number of cytotoxic T cells ($CD8^+$ TCR^+). It is only later in infection that large numbers of B cells, in follicular arrangements, are seen (Gaunson et al. 2006b). In the early stages of infection, this lymphoid accumulation seems likely to be a consequence of the effect of mycoplasma lipoproteins on host macrophages, mediated by the release of proinflammatory chemokines. In the later stages, when B cell proliferation is dominant, antigenic variation in the lipoproteins may cause chronic lymphoid stimulation. A similar pattern is observed in the mammary glands of goats infected with *M. agalactiae* (Castro-Alonso et al. 2008) or cattle infected with *M. bovis* (Kauf et al. 2007), with a rapid initial accumulation of neutrophils and macrophages and secretion of cytokines into the udder. A moderate antibody response occurs later (Castro-Alonso et al. 2008). These responses reduce the concentration of mycoplasmas but do not eliminate them, resulting in chronic mastitis and long-term shedding.

Studies on *M. pulmonis* infections have shown that $CD8^+$ T cells play a significant role in the control of the immunopathology associated with disease, as depletion of these cells increases the severity of pulmonary lesions (Jones et al. 2002). In contrast, depletion of $CD4^+$ T cells results in less severe disease, implying that they are responsible for the immunopathology. Notably, depletion of $CD4^+$ and $CD8^+$ cells has no influence on the number of organisms in the lung. However, in mice with a predisposition for asthma, infection with *M. pulmonis* triggers a series of pathophysiological changes linked to an increased T-helper 2 response, which in turn exacerbates bacterial colonization of the airways (Bakshi et al. 2006). Activation of $CD4^+$ and $CD8^+$ lymphocytes, with an apparent skew toward a T-helper 2 response, is also seen in the respiratory tract of calves infected with *M. bovis* (Vanden Bush and Rosenbusch 2003). In contrast, in cattle infected with *M. mycoides* subsp. *mycoides* small colony type, there is a T-helper 1 response, with an increase in $CD4^+$ lymphocyte activation in animals developing chronic pleuropneumonia (Totte et al. 2008). A T-helper 1 response, characterized by the production of interleukin 12 and interferon gamma, also occurs during infection of pigs with *M. hyopneumoniae* (Rodriguez et al. 2007).

The macrophages and neutrophils recruited at the site of infection produce oxidative bursts that may kill mycoplasmas, with superoxides reacting with cellular iron to form molecules that can damage DNA. Mycoplasmas appear to adapt rapidly to these situations. In *M. hyopneumoniae*, exposure to oxidative stress leads to the up-regulation of 13 genes and the downregulation of 25 genes. While the functions of these genes need to be studied in detail, a gene encoding a putative iron-binding protein, NapA, is among those downregulated (Schafer et al. 2007).

Mycoplasma lipoproteins stimulate other cells, including osteoclasts, which are derived from the monocyte line. This stimulation increases bone resorption *in vitro*, and thus may play a role in arthritides caused by mycoplasmas (Novak et al. 1995; Piec et al. 1999).

Tissue Necrosis

The acute phase of disease caused by *M. mycoides* subsp. *mycoides* small colony type is characterized by an atypical host response, with infiltration of large numbers of neutrophils, deposition of fibrin, extensive edema, necrosis, and abscessation, with the necrotic areas eventually encapsulated by a dense layer of connective tissue. This acute response

appears to be induced by a diffusible toxin, possibly capsular galactan, which causes tissue necrosis.

Microbial Interactions

Synergistic interactions with other infectious agents is a common occurrence in many mycoplasmoses, and particularly respiratory diseases. Interactions between mycoplasmas and viral agents may result in more severe manifestations of the viral disease or of the mycoplasmal disease. For example, *M. hyopneumoniae* induces the production of proinflammatory cytokines leading to inflammatory changes in the lung that diminish the capacity of the immune system to control other respiratory pathogens, including porcine reproductive and respiratory syndrome (PRRS) virus (Thacker et al. 1999), thereby exacerbating pneumonic lesions and contributing significantly to the PRRS disease complex (Thacker 2001). The lesions may be exacerbated further by secondary infection with *Actinobacillus pleuropneumoniae* or with opportunistic pathogens such as *Pasteurella multocida*, *Bordetella bronchiseptica*, and *M. hyorhinis*. In calves, *M. bovis* is often isolated from lung lesions that also contain *Mannheimia haemolytica* (Gagea et al. 2006), and synergistic interactions between these species are suspected to exacerbate disease (Rice et al. 2007).

Mycoplasmal colonization of the epithelial surface can also be facilitated by prior damage caused by viral infection. For example, infectious bronchitis virus (IBV) and Newcastle disease virus infections in chickens predispose to more severe infections with *M. gallisepticum* and *M. synoviae*. Some of these interactions can have remote consequences in the host. For instance, coinfections of the respiratory tract of chickens with *M. synoviae* and IBV increase the incidence of mycoplasmal arthritis (Landman and Feberwee 2004).

Hemotrophic mycoplasmas also appear to be involved in coinfections, as shown by the frequent association of Candidatus *Mycoplasma turicensis* with Candidatus *Mycoplasma haemominutum* (Peters et al. 2008), and *M. haemofelis* and feline leukemia virus or feline immunodeficiency virus (Sykes et al. 2008), in cats.

Influence of Environmental Factors

Environmental factors have a significant influence on many mycoplasmal diseases. Of particular importance has been the intensification of animal production. High stocking densities induce physiological stress that lowers host resistance to infection and favors transmission from animal to animal, thereby increasing the infecting dose. The severity of porcine enzootic pneumonia, caused by *M. hyopneumoniae*, varies substantially with standard of management, season, ventilation, stocking density, and other environmental factors. When husbandry is good, disease in the absence of secondary infection is mild and may have negligible effects on growth rate and mortality (Buddle 1985). A similar effect of environment occurs with *M. gallisepticum* and *M. synoviae* in poultry, and with *M. bovis* and *M. dispar* in intensively reared calves. Herd size is an important epidemiological factor in bovine mycoplasmal mastitis, which is mainly a problem in very large herds.

Environmental stress and pollutants such as ammonia and nitrites increase susceptibility to mycoplasmosis. This may be through a detrimental effect on the mucosal lining or through impaired macrophage function and activity of NK cells. Nutritional factors may also play a significant role in the outcomes of infection. The reduction in egg production caused by infection with *M. gallisepticum* can be ameliorated by supplementation of the diet with higher concentrations of fat (Peebles et al. 2003).

Age has an important influence on the severity of a number of mycoplasmoses. For example, more severe pneumonia at slaughter is correlated with infection with *M. hyopneumoniae* prior to weaning (Fano et al. 2007), and much more severe disease is seen in chickens infected with *M. gallisepticum* when they are less than 4 weeks of age (Gaunson et al. 2006a).

The severity of anemia caused by the hemotrophic mycoplasmas is significantly affected by splenectomy, presumably because of less efficient removal of infected erythrocytes.

PROTECTIVE IMMUNITY

Natural Immunity

Innate immunity is important in determining the outcome of the initial interaction between pathogenic mycoplasmas and their hosts by helping confine the organisms to their natural ecological niches as mucosal parasites of upper respiratory or lower urogenital tracts. The result is that many mycoplasmoses are subclinical and the only evidence of their occurrence may be seroconversion,

unless environmental stressors or other infectious agents reduce the efficacy of this first line of defense.

There is an early inflammatory response to infection, with an influx of neutrophils, macrophages, and NK cells. Alveolar macrophages are the most important first line of defense in protecting the lungs of mice against *M. pulmonis* (Hickman-Davis 2002). The unique diacylated structure of mycoplasma lipoproteins is recognized by a heterodimer of TLR-2 and TLR-6, resulting in potent activation of macrophages (Muneta et al. 2003; Rharbaoui et al. 2004). Opsonins, including complement, are also thought to be important for phagocytosis and killing of mycoplasmas, as are the surfactant proteins SP-A and SP-D and reactive free radicals (Hickman-Davis 2002). Mast cells also appear to play significant roles in reducing replication of *M. pulmonis* in the lungs of infected mice (Xu et al. 2006a), whereas NK cells appear to promote the inflammatory response, but not clearance of mycoplasmas, unless this effect is countered by production of interferon gamma (Woolard et al. 2005).

In the early acute stages of experimental infection with virulent *M. gallisepticum* in the chicken, $CD8^+$ TCR^- lymphocytes (believed to be the avian homolog of mammalian NK cells) infiltrate the tracheal mucosa and are arranged in follicle-like aggregates (Gaunson 2001; Gaunson et al. 2006b). However, whether they play a role in killing mycoplasmas or in influencing the inflammatory response is yet to be established.

Mycoplasmas induce potent proinflammatory cytokine responses, usually with a pronounced T-helper 2 bias. This response has been seen in respiratory tract infections and also in mammary gland infection with *M. bovis* (Kauf et al. 2007), but is less evident in respiratory tract infections with *M. gallisepticum* (Mohammed et al. 2007). While mycoplasmas induce production of cytokines that result in significant influxes of polymorphonuclear and mononuclear leukocytes, there is increasing evidence that they also have immunosuppressive effects. Infection with *M. gallisepticum* causes a significant decrease in CCL20, interleukin 8, and interleukin 12 gene expression very early in infection (Mohammed et al. 2007), and *M. gallisepticum* has a significant systemic immunosuppressive effect that can be counteracted by interferon gamma (Muneta et al. 2008). While innate immunity plays a role in resistance, in naturally occurring infections in immunologically naïve animals it may be insufficient to prevent disease. However, animals that have previously been exposed to a specific mycoplasma infection can show a high level of resistance on reinfection, indicating a role for adaptive immunity in protection.

Local adaptive immune mechanisms appear to be an important line of defense. While the precise mechanism(s) of protection remain to be determined, neonatally bursectomized chickens show increased susceptibility to *M. gallisepticum* infection (Mukherjee et al. 1990), suggesting a key role for antibody. As there is no correlation between levels of serum antibody and protection (Whithear 1996), it is more likely that local antibodies are more important. Mycoplasma lipoproteins have been shown to stimulate maturation and enhance antigen processing and presentation by dendritic cells. Infection of chickens with *M. gallisepticum* stimulates mucosal proliferation of B lymphocytes during the first week and the tracheal mucosa has significantly increased concentrations of both IgA and IgG secreting plasma cells, resulting in higher concentrations of mucosal IgA and IgG against *M. gallisepticum* (Javed et al. 2005; Gates et al. 2008). IgA and IgG may prevent the attachment of mycoplasmas to mucosal epithelial cells, while IgG may opsonize organisms, thus enhancing phagocytosis. Antibody is also important in preventing dissemination of infection to extrapulmonary sites by *M. pulmonis* (Cartner et al. 1998).

The nature of the cellular response in mycoplasma infected lung tissue (i.e., perivascular and peribronchiolar accumulations of small mononuclear cells) suggests that T cells may be involved in response to and recovery from infection. However, direct experimental evidence is lacking and cell-mediated immunity appears to be of limited importance in protection (Cartner et al. 1998). In summary, it would appear that both innate and adaptive mechanisms are important in immunity to mycoplasma infections. In keeping with the heterogeneity of mycoplasmas, it is likely that immunity to mycoplasmosis will prove to be a complex interaction of a range of responses that could differ for individual mycoplasmas.

The immune responses also play a major role in the development of the lesions characteristic of mycoplasmosis. T lymphocyte-dependent antibody responses are responsible for the angiogenesis, lymphangiogenesis, and epithelial remodeling seen in chronic inflammatory responses to *M. pulmonis* in

mice (Aurora et al. 2005). Histamine production by neutrophils can be a significant contributor to the severity of inflammation during acute mycoplasmosis (Xu et al. 2006b).

Artificial Immunity

Some mycoplasmoses have been successfully controlled by vaccination including, notably, *M. mycoides* subsp. *mycoides* small colony type in cattle, *M. hyopneumoniae* in pigs, and *M. gallisepticum* and *M. synoviae* in poultry. Louis Willems was the first to demonstrate the efficacy of immunization against mycoplasmosis (Huygelen 1997). He inoculated susceptible cattle in the tip of the tail with serous fluids from affected cattle and showed that they were protected against subsequent exposure to infection. Since then researchers have developed various live attenuated strains of *M. mycoides* subsp. *mycoides* small colony type that produce much less severe local reactions at the injection site than infected tissue fluids or cultures of fully virulent strains. Bacterins have failed to confer protection and may even sensitize cattle, resulting in a more severe reaction to subsequent challenge (Gourlay 1975).

Bacterins are commercially available for the control of porcine enzootic pneumonia (Jensen et al. 2002). These vaccines stimulate systemic humoral responses, but not local antibody or cell mediated immunity. They are reported to reduce the incidence of lesions but do not prevent infection, and experimental studies suggest that they do not prevent transmission to any significant extent (Meyns et al. 2006). Paradoxically, there is no correlation between serum antibody and protection with *M. hyopneumoniae* (Etheridge and Lloyd 1982). Protection has also been induced experimentally in pigs vaccinated with cell-free culture supernatant (Okada et al. 2000). A bacterin has also been reported to protect against pneumonia induced by experimental challenge with *M. bovis* (Nicholas et al. 2002).

Bacterins have also been used against *M. gallisepticum* and *M. synoviae* but have been superseded by live attenuated vaccines. Experimental studies have shown that attenuated vaccines against *M. gallisepticum* are more effective than bacterins in preventing transmission (Feberwee et al. 2006a, 2006b). For example, strain ts-11 (Whithear et al. 1990), a highly attenuated temperature sensitive mutant of *M. gallisepticum*, has been used successfully in many countries to control *M. gallisepticum*. Unlike pathogenic *M. gallisepticum*, vaccination with ts-11 does not stimulate an influx of T cells into the tracheal mucosa, and chickens vaccinated with ts-11 do not develop such a response following challenge (Gaunson et al. 2006b). Furthermore, systemic antibody concentrations have little correlation with protection after vaccination with this attenuated vaccine (Noormohammadi et al. 2002). Vaccination of birds under 4 weeks of age is less effective, but still prevents severe disease (Gaunson et al. 2006a). Temperature-sensitive mutant vaccines have also been developed and used widely to protect chickens against *M. synoviae* (Markham et al. 1998a; Morrow et al. 1998; Jones et al. 2006a, 2006b, 2006c; Noormohammadi et al. 2007), and a similar vaccine has also been reported to protect rats against *M. pulmonis* (Lai et al. 1991).

Newer Approaches to the Development of Mycoplasma Vaccines

Current commercially available vaccines for use in animals have been empirically developed because of lack of knowledge of the identity of protective immunogens, the mechanisms of protective immunity and the key factors involved in virulence. However, significant advances are being made toward development of defined attenuated mutants by deletion or disruption of virulence genes in some species, including *M. gallisepticum* (Gates et al. 2008) and *M. mycoides* subsp. *mycoides* small colony type (Janis et al. 2008). Initial steps are also being made in the use of attenuated vaccine strains of mycoplasmas as vectors to deliver antigens of other pathogens or to deliver cytokines to mucosal surfaces (Muneta et al. 2008). Although there have been a number of studies of immune responses to selected purified mycoplasma proteins, the limited success of bacterins and the prevalence of immune evasion mechanisms in mycoplasmas suggest that subunit vaccines are unlikely to be as effective as attenuated vaccines.

CONCLUSIONS

The pathogenesis of mycoplasmosis continues to be an area of active research, with most emphasis on furthering understanding of the mechanisms involved in antigenic variation, motility, and adherence; the significance of antigenic variation; and the immune responses to infection. While antigenic variation has been characterized in some detail in several pathogenic species, and in many cases

assumed to play a role in persistence, definitive proof of its significance is lacking. Some of the proteins involved in motility have been defined, but the functional details of mycoplasma motility are yet to be fully explained. An increasing focus is being applied to the nature of the immune response, the cytokines involved in the generation of the lymphoid response, and the crucial effectors of protective immunity. Future studies of adherence will provide further detail of the structure of the attachment organelle, and identify adhesins used by other pathogenic species, some of which are likely to be novel proteins, rather than homologs of those already known.

The genomes of many of the most pathogenic mycoplasmas have been fully characterized in recent years and attention is now being focused on determining the role of genes of unknown function in these pathogenic mycoplasmas, with a particular emphasis on identifying virulence genes. Lipoprotein multigene families have been found in the genomes of the fully sequenced mycoplasmas, but in most cases the functions of the lipoproteins they encode are not known, and the mechanisms used by the organisms to control expression of different family members is not obvious. A further area of research that is likely to yield considerable understanding of the evolution of virulence in mycoplasmas is the investigation of the mechanisms involved in the massive horizontal transfer that has been detected in some of the avian and ruminant mycoplasmas.

Finally, as the taxonomic position of the hemophilic mycoplasmas has been clarified only recently, the molecular pathogenesis of these species has only just begun to be studied in any detail. Given the atypical habitat of these pathogens, it is likely that details of the pathogenesis will be found to differ significantly from those of the other mycoplasmas.

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30
Chlamydia

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INTRODUCTION

Chlamydiae are gram-negative, obligate intracellular bacteria with a unique developmental cycle, consisting of the elementary body (EB), the condensed infectious form, and the reticulate body (RB), the larger replicating form. They infect a broad range of species, including humans, and are widespread in nature. Chlamydiae cause economically important diseases in farm animals, including ocular, pulmonary, genital, articular, and intestinal infections; these are often persistent, chronic, or subclinical, and are difficult to diagnose and treat. Chlamydiae depend on host cells for multiplication.

CLASSIFICATION

The genus *Chlamydia* was proposed in the 1940s and validated by combination with a specific epithet to establish the type species *Chlamydia trachomatis*. The Approved List of Bacterial Names in 1980 included only *C. trachomatis* and *Chlamydia psittaci* in the order Chlamydiales (Storz and Page 1971). *C. trachomatis* accumulated glycogen in inclusions and were sensitive to sulfadiazine, while *C. psittaci* had the opposite pattern. Establishment of this classification was a milestone in chlamydial taxonomy, as it renounced reliance on presumed host, presumed tissue preference, and serology (Everett et al. 1999). *Chlamydia pneumoniae* and *Chlamydia pecorum* were added (Herring 1993). However, DNA sequence analysis led to the division of the Chlamydiaceae into genera *Chlamydia* and *Chlamydophila*, comprising a total of nine species (Everett et al. 1999). The genus *Chlamydia* now includes *C. trachomatis* (human), *Chlamydia suis* (swine), and *Chlamydia muridarum* (mouse, hamster), while the genus *Chlamydophila* includes *Chlamydophila pecorum* (ruminants), *Chlamydophila pneumoniae* (human, koala, horse, amphibian), *Chalmydophila psittaci* (avian), *Chlamydophila abortus* (ruminants), *Chlamydophila felis* (cats), and *Chlamydophila caviae* (guinea pigs). This was based upon sequences of 16S and 23S rRNA and *omp*A (which encodes the major outer membrane protein [MOMP]), and genes encoding GroEL chaperonin, KDO transferase, small cysteine-rich lipoprotein, and 60-kDa cysteine-rich protein (*omp*B).

The revised taxonomy has been widely accepted by chlamydiologists, especially in veterinary medicine, by virtue of the pratical value of its classification of host range and clinical disease. However, it still faces opposition from some in the human chlamydia research community who contest the division into two genera (Schachter et al. 2001; Stephens 2008).

Chlamydia-like bacteria have been discovered and characterized, and three new families in order Chlamydiales (Parachlamydiaceae, Simkaniaceae, and Waddliaceae) have been proposed (Everett et al. 1999). The order Chlamydiales now contains at least eight recognized families, and molecular evidence suggests an additional, yet-unexplored diversity of environmental chlamydiae, which carry out their obligate intracellular lifestyle as symbionts of free-living one-celled organisms. They infect various cell types of diverse eukaryotic hosts, and may be of medical relevance for humans and animals (Corsaro and Greub 2006; Horn 2008).

HOST–PARASITE RELATIONSHIP: CLINICAL DISEASE AND PATHOGENESIS

Severity of chlamydial infections vary from clinically-inapparent to severe and systemic. The latter are characterized by fever, anorexia, lethargy, and occasionally, shock and death. An asymptomatic carrier state or persistent infection is a common sequel if the infection is not treated. Chlamydiae can be shed in feces, urine, respiratory secretions, amniotic fluid, and placentae of symptomatic or asymptomatic animals, and in saliva or feather dust of birds. Strain virulence, site of infection, and the number of bacteria shed significantly impact the likelihood of transmission.

Bacteria in the order Chlamydiales infect a broad range of species. Most are agents of animal and/or human diseases (Storz and Kaltenboeck 1993). *C. trachomatis* is the leading cause of preventable blindness and the most common agent of sexually-transmitted diseases in humans (Schachter 1999). *C. pneumoniae* causes acute and chronic respiratory infections in humans, and has been implicated in cardiovascular disease. Strains of this organism have been identified in reptiles (snakes, iguanas, chameleons, turtles, crocodiles), amphibians (frogs), horses, koalas (Bodetti et al. 2002; Soldati et al. 2004; Blumer et al. 2007), and Australian marsupials (Kutlin et al. 2007). Human (also called TWAR, derived from TW-183 and AR-39), equine, and koala biovars have been described (Everett 2000). The koala nasal *C. pneumoniae* isolate (LPCoLN) is by far the best genetically characterized, and recently, *in vitro* key differences in doubling time, size, and morphology of this strain were compared to human isolate AR39 (Mitchell et al. 2008). In conclusion, strains infecting humans, unlike those of animals, are very similar and current human strains may have evolved relatively recently from an unknown animal strain (the horse strain is the closest strain to human). However, the zoonotic potential of *C. pneumoniae* in animals remains unclear.

C. psittaci, C. abortus, *C. pecorum*, and *C. suis* have been associated with disease in production agriculture. *C. psittaci* primarily infects birds, and all avian species are considered susceptible. It has been found in all major domestic fowl and in 460 free-living or pet bird species representing 30 orders (Kaleta and Taday 2003). Psittacine birds, turkeys, ducks, and pigeons are the most commonly reported sources of infection in humans. Chickens are highly resistant but not insusceptible.

There are six serovars of *C. psittaci*, based on MOMPs (Andersen 1997). These serovars are congruent with the genotypes defined by *omp*A polymorphisms. The current genotyping scheme comprises seven avian (A, B, C, D, E, F, E/B) and two nonavian (WC, cattle; M56, muskrat) genotypes (Vanrompay et al. 1997; Geens et al. 2005). M56 and WC were isolated from single outbreaks. Most recently, subtypes of the more heterogeneous genotypes A, E/B and D, as well as six new provisional genotypes representing untypable strains, have been detected by DNA microarray assays (Sachse et al. 2008) and variable number of tandem repeats (VNTR) analysis (Laroucau et al. 2008). Serovars A and B are usually endemic among psittacine birds and pigeons, respectively. Natural hosts of the other serovars are less certain. Serovar C has been obtained primarily from ducks and geese but may have been transmitted from pet birds to a dog (Sprague et al. 2008). Serovars D and F are mainly from turkeys. The host range of serovar E has the most diverse host range, which includes pigeons, ratites, ducks, turkeys, and humans. Genotype E/B has been isolated mainly from ducks. *C. psittaci* has also been associated with recurrent airway obstruction in horses (Theegarten et al. 2008) and abortions in pigs (Kauffold et al. 2006). All serovars are readily transmissible to humans (Vanrompay et al. 1993, 1997; Geens et al. 2005).

Chlamydophila psittaci

Chlamydiosis is common in psittacine birds and is a major zoonosis. Disease severity in birds largely depends on the affected bird species and the virulence of the chlamydial strain. *C. psittaci* strains can cause systemic disease in domestic poultry and wild fowl, as well as psittacines, but many birds show no clinical signs until stressed. Intermittent shedding is common, and this is a source of infection for other birds and humans. Infections cause enteritis, air sacculitis, pneumonitis, and hepatosplenomegaly. Chlamydiosis in pigeons is similar to that in psittacine birds; chronic infections lead to the asymptomatic carrier state. However, signs of disease are more likely to be conjunctivitis, blepharitis, and rhinitis. Chlamydiosis in ducks is a serious economic and occupational health problem in Europe. Trembling, conjunctivitis, rhinitis, and diarrhea are common signs, with up to 30% mortality.

Severe spontaneous outbreaks of *C. psittaci* in turkeys in the United States has an associated case fatality rate of 5–40% without treatment (Andersen 1996). Postmortem findings in natural infections are vasculitis, pericarditis, splenitis, and nasal adenitis. Birds inoculated with virulent turkey strain TT3 experienced a 20%–40% decrease in body weight, and pericarditis was the most severe lesion. Air sac lesions were less severe, and there was no evidence of bronchopneumonia. Microscopic lesions were found in the lateral nasal glands, and colonization of this site could facilitate transmission by aerosol.

Disease is probably introduced into flocks by feral birds (Harkinezhad et al. 2008). Bacteriologic and serological data support a hypothesis of secondary transmission to contacts following inoculation of birds by the oral route. On the other hand, aerosol exposure as the primary means of exposure is supported by findings that chlamydiae are first isolated from the oropharyngeal and nasal secretions, and that survival in feces is brief. Chlamydiae are rapidly disseminated throughout the body following aerosol exposure, and are recovered from the lung, air sacs, pericardial sac, and mesentery within 4 h. The virulent turkey strain causes little or no disease in chickens, pigeons, or sparrows, but cockatiels and parakeets succumb rapidly. The mortality rate in turkeys can be 10–30% without early antibiotic treatment.

Transmission generally occurs via infectious dust and aerosols, through drinking water, through *in ovo* infection, or from the parent to the young birds by regurgitation at the time of feeding (Harkinezhad et al. 2008). *C. psittaci* can also be transmitted in nests by lice, flies, and mites (Longbottom and Coulter 2003).

Human infection with *C. psittaci* can also occur following exposure by inhalation or ingestion. Symptoms are variable and nonspecific, ranging from mild flu-like illness with fever, headache, myalgia, and diarrhea, to endocarditis, encephalitis, and severe, sometimes fatal, pneumonia.

C. abortus causes enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA) and is the most common infectious abortigenic agent worldwide in small ruminants. The organism cause significant economic losses in Europe, North America, and Africa. Ewes and goats experience late term abortions, stillbirths, or delivery of weak lambs or kids. Cattle, pigs, deer, and horses can also be infected. Abortions are due to placental infection; most occur during the last month of gestation, but may occur as early as the 100th day of gestation. Clinical signs are rare in the dam, and are usually limited to vaginal discharge, which is more frequent in goats than in ewes.

Immunologically naïve flocks experience abortion rates of 30% in ewes and up to 60% in goats. This persists for 2–3 years, at which time most females are infected. Enzootic abortion rates are 1–5% until an abortion storm involving all primiparous females occurs. This cyclic behavior of the agent is due to lifelong immunity that follows abortion (Rodolakis et al. 1998). Second abortions are rare. Aborting females recover rapidly; placental retention is rare in ewes but can occur in both goats and cows and may result in endometritis.

Sheep become infected by ingesting and inhaling *C. abortus* directly from aborted placentae or vaginal discharge, or indirectly via contaminated food. The organism was found in semen of bulls, rams, and bucks, but veneral transmission is not of great importance (Teankum et al. 2006). Infection is first established in the tonsils (Jones and Anderson 1988), from which it disseminates via blood to other organs. The organism then persists latently, and intermittent low-grade chlamydiosis occurs, eventually infecting the placenta (Huang et al. 1990). Placental infections usually become established sometime between 60 and 90 days of gestation, with pathological changes first detected after 90 days (Buxton et al. 1990). The organism was detected in the placenta as early as 15 days after inoculation in ewes inoculated subcutaneously at gestation day 70 (Maley et al. 2008). Microscopic lesions appeared in the placenta at 25 days post infection (Buxton et al. 1990; Netleton and Entrican 1995; Maley et al. 2008). The infection remains asymptomatic in open females or those infected late in pregnancy, and may induce abortion in the next gestation (Wilsmore et al. 1984). Polymerase chain reaction (PCR) detection of *C. abortus* is rare in post-abortion ewes at estrus and subsequent lambing (Livingstone et al. 2008). The mechanism by which chlamydiae migrate from the maternal side of the placenta to the fetus is uncertain. However, at about 60 days' gestation, maternal hematomas appear in the placentomal hilus as a result of normal invasion of the caruncular stroma by fetal chorionic villi (Sammin et al. 2008). This could allow any circulating *C. abortus* to have direct contact with the chorionic epithelium (Buxton

et al. 1990). Endocrinologic and/or immune-related changes may play a significant role in lesion formation (Buxton et al. 1990; Maley et al. 2008; Sammin et al. 2008). Progression of the infection results in a considerable loss of chorionic epithelial cells in both the cotyledonary and intercotyledonary placenta, and there is a mixed-cell inflammatory infiltrate. Fibrin deposits and a purulent arteritis are noted in the thickened placental mesenchyme underlying the lesions. Chlamydial inclusions are present in the endometrial epithlium, where affected chorion is in apposition to the maternal tissues. Severe necrosis and sloughing of the endometrial epithelium occur in later stages (Maley et al. 2008).

Fetal infection is secondary to placentitis. Necrotic foci, sometimes with inflammatory reactions, are frequently found in most fetal tissues, and may be embolic (Buxton et al. 1990). Chlamydial antigen, if present in the foci, is usually in small amounts. The popliteal and mesenteric lymph nodes of infected fetuses are usually enlarged, having a demarcated cortex with several follicles and germinal centers.

C. abortus has been isolated from cervical swabs and genital tracts of sows with repeat estrus, abortions, and small litters with weak piglets (Thoma et al. 1997; Hoelzle et al. 2000; Camenisch et al. 2004). However, *C. suis* and *C. pecorum* are more common in porcine abortions.

C. abortus presents a zoonotic risk, rarely inducing mild flu-like symptoms or pneumonia in humans, but occasionally causing severe illness and abortion. Fever, headache, nausea, and vomiting are generally the first symptoms, followed by abortion and, if untreated, severe complications such as acute renal failure, disseminated intravascular coagulation, or respiratory distress necessitating mechanical ventilation. Pregnant women are advised to stay away from sheep during lambing.

C. pecorum has been associated with sporadic cases of encephalomyelitis, polyarthritis, conjunctivitis, enteritis, mastitis, pneumonia, and reproductive disorders in ruminants, pigs, and koalas. Ovine chlamydial polyarthritis of lambs affects synovial tissues involving most limb joints. Affected ruminants have varying degrees of stiffness, lameness, and anorexia. Polyarthritis in sheep and cattle is readily reproduced by oral, intramuscular, subcutaneous, intravenous, or intraarticular inoculation.

Under field conditions, the organism is ingested and subsequently multiplies in the mucosa of the large and small intestines, sometimes causing diarrhea and leading to chlamydemia. Periarticular and articular tissue changes are seen. Periarticular effects include joint enlargement, and these enlarged joints have excessive greyish-yellow, turbid synovial fluid. Fibrin plaques are also found in joints with advanced lesions. In severe cases, tendon sheaths may also contain excess fluids. Muscle involvement is limited to the point of tendinous attachment. Microscopic changes are primarily an inflammatory reaction in the synovium, tendon sheaths, and subsynovial tissues (Shupe and Storz 1964; Cutlip and Ramsey 1973). Granulation tissue replaces much of the fibropurulent exudate in inoculated joints, with the formation of large fibrous villi by ~21 days. By this time, synovial surfaces will again be covered by intact lining cells.

C. pecorum can also cause pneumonia, conjunctivitis, enteritis, encephalomyelitis, urinary tract disease, metritis, and fertility disorders in small ruminants, cattle, swine, and koalas. However, most strains of *C. pecorum* are not considered highly virulent, and most healthy ruminants harbor the organism, without symptoms, in the intestines.

C. pecorum has many antigenic and genomic variations, particularly in the MOMP (Denamur et al. 1991; Salinas et al. 1995; Kaltenboeck et al. 2008). Differential identification of virulent *C. pecorum* strains by multivirulence locus sequence typing, based on the analysis of the *omp*A, incA, and ORF663 genes, may be useful in epidemiological investigations (Yousef Mohamad et al. 2008).

C. suis has been identified recently in swine, associated with respiratory disease, enteritis, and conjunctivitis. Distinct strains have been isolated, some of which produce severe disease in gnotobiotic piglets (Rogers and Andersen 1996; Rogers et al. 1996). Results of several field surveys suggest that most intestinal chlamydial infections are asymptomatic (Pospischil and Wood 1987), but pigs with enteritis and/or pneumonia are more likely than normal pigs to be infected (Hoelzle et al. 2000). Tetracycline-resistant strains have been isolated in the United States and Europe (Lenart et al. 2001; Di Francesco et al. 2008). There is molecular evidence of *C. suis* in wild boars, frogs, and sheep (Hotzel et al. 2004; Blumer et al. 2007; Polkinghorne et al. 2008).

C. caviae is isolated from guinea pigs with conjunctivitis (Lutz-Wohlgroth et al. 2006), and this is a widely used experimental model of *C. trachomatis* ocular and genital infection in humans. *C. caviae*

infections may be asymptomatic infection or produce clinical signs ranging from mild to severe conjunctivitis, mucopurulent ocular discharge, chemosis, follicular hypertrophy, and pannus formation. Genital infections leading to abortion or chronic reproductive tract infection compare to chronic infection with *C. trachomatis* in women.

C. felis is associated with severe conjunctivitis in cats (von Bomhard et al. 2003), accompanied by blepharospasm, conjunctival hyperemia, chemosis, ocular and nasal discharges, and fever (Wills et al. 1987). In addition, *C. felis* colonizes the gastrointestinal (GI) and reproductive tracts, producing chronic salpingitis in the latter. A role in feline pneumonia has not been confirmed (Bart et al. 2000). Transmission is by direct contact with infected secretions. Signs usually appear after an incubation of 4 days, and the clinical course is ~30 days. Persistent infections maintain the agent in a population. It has been recovered from eyes, vagina, feces, and superficial gastric mucosa for >150 days post infection (Wills et al. 1987). *C. felis* also causes human conjunctivitis (Yan et al. 2000).

Chlamydia-like bacteria occur as symbionts of free-living amoebae and other eukaryotic hosts. Initially regarded exclusively as environmental organisms, some have medical relevance. Recently, *Parachlamydia* and related organisms have been associated with ruminant abortion (Borel et al. 2007; Ruhl et al. 2008a, 2008b). *Waddlia chondrophila* has been isolated from aborted bovine fetuses in the United States and Europe (Rurangirwa et al. 1999; Henning et al., 2002), and anti-*Waddlia* antibodies were found in women who had miscarried (Baud et al. 2007). Species of two novel families, the Piscichlamydiaceae and the Clavochlamydiaceae, were identified in gill tissues of fish with epitheliocystis (Draghi et al. 2004; Karlsen et al. 2007).

LIFE CYCLE

Chlamydiae are obligate, intracellular bacteria that multiply in the cytoplasm of eukaryotic cells. They depend on the cell for energy and most nucleotide-metabolizing enzymes. The growth cycle consists of EBs, a condensed form (200–300 nm in diameter) suited to survival outside the cell, and RBs (500–1000 nm in diameter) that replicate by binary fission in cytoplasmic inclusions; the latter predominates during most of the life cycle. Variably sized intermediate forms are called dispersing forms or condensing forms, depending on whether transit is from EB to RB or vice versa.

Considerable diversity exists in the ultrastructure of the developmental forms (Horn 2008). Another form, the crescent body, observed in *Parachlamydia acanthamoebae*, is considered an infectious stage. Some isolates of chlamydia-like bacteria have been observed to reside directly in the host cell cytoplasm.

EB attachment and entry into a cell are followed by transition into a metabolically active RB and avoidance of destruction by prevention of endosome acidification and phagolysosome fusion. RB replication depends upon cellular components, without destroying host cellular functions, and is followed by maturation of noninfectious RBs into infectious EBs. Release of EBs, by lysis or exocytosis, and transfer to new cells complete the cycle (Ward 1988). The cycle is similar in chlamydia-like bacteria, although there are differences in stages, subcellular locations, and outcome (Horn 2008).

ABERRANT DEVELOPMENT CYCLE

Persistent or chronic infections play a role in pathogenesis of many chlamydial diseases. An alternative developmental stage, with abnormally sized, enlarged RB-like structures (called aberrant bodies [ABs]), occurs during persistent infection (Campbell et al. 1993). Characterization of ABs has defined chlamydial persistence as the occurrence of viable but noncultivatable intracellular stages (Hogan et al. 2004). Persistent stages can be induced *in vitro* by adding penicillin, ampicillin, or cycloheximide to media, infection with *Chlamydia* phage G1, deprivation of nutrients (e.g., iron and tryptophan), exposure to cytokines such as IFNγ, and coinfection with other agents (e.g., herpes simplex virus type 2) (Deka et al. 2007). Chlamydiae used in these experiments include *C. abortus* (Entrican et al. 2004), *C. psittaci*, *C. trachomatis*, *C. pneumoniae*, and *C. caviae* (Hogan et al. 2004).

Mechanisms of *in vivo* persistence are ill defined and documentation of persistent infection is more difficult, mainly due to the difficulty of unequivocal identification of persistent stages in tissues (Ward et al. 1990; Deka et al. 2007). One study did reveal *C. suis*-associated ABs in intestinal tissues of pigs (Pospischil et al. 2008) (fig. 30.1). Differential gene expression during persistence

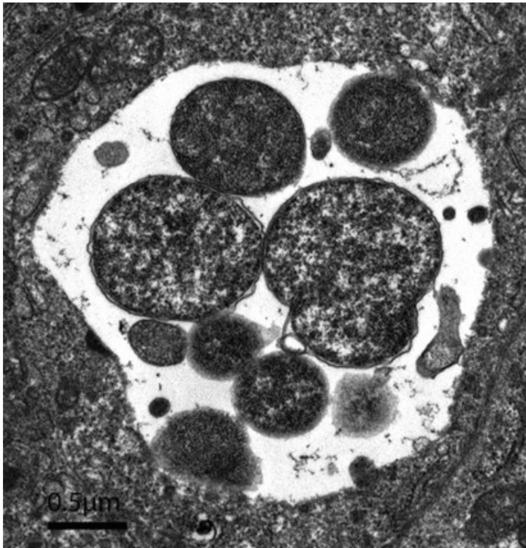

Figure 30.1. Ultrastructure of chlamydial reticulate and aberrant bodies in a chlamydial inclusion.

may allow identification of persistent stages *in vivo* (Molestina et al. 2002).

HOST RESPONSE

Chlamydiaceae drive important parts of the host response, especially in that they must preserve their host cell for replication (Haecker et al. 2006). Knowledge of these mechanisms is crucial for the design of antichlamydial vaccines (Entrican et al. 2001). Most work has been with *C. trachomatis*, *C. pneumoniae*, and *C. abortus* (Stephens 2003; Entrican et al. 2004; Haecker et al. 2006).

Host response to chlamydial infections was for many years investigated under an "immunological paradigm" (Stephens 2003). Potent, rapid TH1 and delayed-type hypersensitivity (DTH) responses are important for rapid clearance of chlamydial infection and prevention of latency in mice and guinea pigs (Wang et al. 1999; Igietseme et al. 2000). Lack of such responses leads to persistent infection, with a low-grade chronic immune response that causes tissue damage. Immune responses can be modulated through immune evasive mechanisms, affecting apoptosis or downregulating MHC class I and II antigen expression (Zhong et al. 2000).

Persistent infection with *C. trachomatis* causes chronic inflammation and the scarring that accompanies eyelid inversion in trachoma or fallopian tube constriction in infertility. This host response has been viewed as antigen-dependent DTH or autoimmunity, but it is now hypothesized that persistent infections of nonimmune epithelial cells result in secretion of proinflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8, IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF). These cause an influx of polymorphonuclear leukocytes (PMN), T cells, B cells, and macrophages, resulting in necrosis and scar formation (Stephens 2003). In fact, inflammation in chlamydial infections is dominated by the activity of cytokines. IFNγ exerts antichlamydial activity *in vivo* and may be important in controlling early primary infections. However, IFNγ restricts the growth of chlamydiae in a dose-dependent manner; low doses restrict growth, medium doses induce persistence, and high doses prevent growth (Entrican et al. 1998; Wang et al. 1999; Hogan et al. 2004). Growth inhibition is by induction of indoleamine 2,3-dioxygenase, the enzyme that decycles tryptophan to N-formylkynurenine (Byrne et al. 1986).

Intracellular chlamydiae are in an occluded membrane not permeable to macromolecules. A type III secretion (TTS) system brings effector molecules into direct proximity of host cell target proteins. The complete repertoire of TTS genes has been identified in *C. trachomatis* and *C. psittaci* (Stephens et al. 1998; Haecker et al. 2006). Hollow spike-like surface appendages traversing inclusion membranes were observed by electron microscopy (Matsumoto 1981) and are likely part of the TTS system.

It has been challenging to identify developmental cycle stages in which the secretion system is active. Current evidence is that some TTS and inclusion proteins are secreted during the putatively dormant EB stage (Fields et al. 2003). Several potential TTS secreted proteins are apparently introduced into the cell early on, but TTS system proteins are only present late in the development cycle, after EBs convert to RBs. Genes for the TTS system may be expressed during the RB stage, the secretion apparatus may be replenished during the RB stage, and these secretion pores may serve newly formed EBs.

Chlamydial infections both stimulate and inhibit apoptosis. During early stages of infection and during persistent infection, recognition of chlamydiae is through Toll-like receptors (TLRs) of macrophages and epithelial cells. Chlamydiae then induce antiapoptotic activity via a unique dual method,

directly attacking the apoptotic pathway via classic activation of NF-κB and at the same time by preventing release of cytochrome C and activation of the caspase machinery (Haecker et al. 2006). This favors completion of the chlamydial replicative cycle (Fan et al. 1998). In contrast, apoptosis is enhanced during late stages of infection (Ojcius et al. 1998), possibly promoting host cell rupture and relase of EBs. However, IFNγ-induced persistent infection leads to resistance to apoptosis, even during late infection stages (Dean and Powers 2001; Perfettini et al. 2002).

The extent to which chlamydiae infect or reinfect hosts suggests that they exploit niches in which they survive the immune response. The response of mucosal epithelial cells to infection is likely the key to subsequent development of adaptive immunity and the pattern of disease progression (Entrican et al. 2004).

Immunity

The immune response to infection eliminates the organism from the body. It provides solid protection from reinfection by the same strain, but subsides after 4–6 months. Immunity is likely both cell mediated and antibody mediated. The role of each may depend on the location of the bacterium in the body (i.e., the eye, respiratory tract, or genital tract). The response to the primary infection includes high levels of antibody to the chlamydial lipopolysaccharide (LPS) or genus-specific epitopes. Immunoassays directed against the chlamydial LPS have been developed to detect chlamydiae; however, they are generally only Chlamydiaceae specific and do not allow the identification of the species, serotype, or subtype involved. Neutralizing protective antibodies are primarily to the MOMP and are likely serovar specific. These are highly immunoreactive proteins comprising epitopes present in at least the *Chlamydophila* species (Longbottom et al. 1996, 1998; Vretou et al. 2003). Antibodies to the polymorphic outer membrane protein (POMP) family can be detected even earlier than anti-MOMP antibodies after experimental infection of pregnant ewes or after hyperimmunization of mice (Vretou et al. 1996; Livingstone et al. 2005).

PREVENTION AND CONTROL

Antibiotic Treatment

Chlamydiae are susceptible to tetracyclines, quinolones, and macrolides, but these are not bactericidial; therapeutic levels must be high and administered over a long period to clear infection and avoid the risk of relapse. In an outbreak of chlamydial abortion in small ruminants, the number of abortions and the shedding of *C. abortus* at parturition was reduced during the last month of pregnancy by injection of tetracycline (Buxton and Henderson 1999). However, infection was not cleared by this treatment and shedding continued, posing a risk for pregnant women.

Doxycycline is generally recommended for treatment of *C. felis* conjunctivitis in cats. Treatment should continue for at least 4 weeks, and oral antimicrobial is more effective than eye ointments (Dean et al. 2005). Infected cats should be isolated.

C. psittaci infection in birds is treated via antibiotics in food or drinking water or by intramuscular injection. The extent of bacterial clearance should be checked at the end of treatment.

Isolation of tetracyline-resistant *C. suis* strains from pigs has been reported (Lenart et al. 2001; Di Francesco et al. 2008) and should be considered in planning antibiotic treatment in pigs.

Vaccination

There are commercial vaccines for prevention of *C. abortus* ovine abortion and for *C. felis* infection. No commercial vaccine is available for avian chlamydiosis, despite intense work on plasmid DNA vaccines for turkeys expressing the MOMP of *C. psittaci* serovar A (Vanrompay et al. 1999, 2001).

Live and inactivated vaccines have been developed for *C. abortus* in sheep. An egg-grown, formalin-inactivated, whole-organism vaccine used before breeding significantly reduces the incidence of abortion (McEwen and Foggie 1954; Jones et al. 1995; Garcia de la Fuente et al. 2004). A live temperature-sensitive mutant vaccine is available in several countries (Rodolakis and Souriau 1983; Chalmers et al. 1997). This attenuated *C. abortus* strain (1B) was obtained from the virulent strain AB7 by nitrosoguanidine mutagenesis (Rodolakis 1983; Rodolakis and Souriau 1983). The live vaccine is more efficient than the inactivated vaccine, inducing the same immunity as natural disease (Rocchi et al. 2008). It is also effective against *C. pecorum* infection (Rekiki et al. 2004), but not against *C. psittaci*. However, vaccinated animals cannot be differentiated from naturally infected animals by serological methods (Borel et al. 2005; Gerber et al. 2007). It

cannot be used in pregnant animals, and precautions must be taken to prevent exposure of pregnant women.

No subunit or recombinant vaccine is as effective as the live vaccine (Longbottom and Livingstone 2006). Plasmid DNA vaccines expressing the MOMP of *C. abortus* either failed to protect mice against challenge (Héchard et al. 2003) or produced lower clearances rates in mice than the live vaccine (Zhang et al. 2008).

Live attenuated and inactivated vaccines are also available for *C. felis*. Live attenuated and experimental inactivated vaccines reduce or eliminate clinical signs but do not completely prevent infection and shedding (Shewen et al. 1980; Wills et al. 1987). A novel transmembrane head (TMH) family protein of *C. felis* has potential as a specific diagnostic antigen for distinguishing infected from vaccinated cats (Ohya et al. 2008).

FUTURE DIRECTIONS

The greatest impact of research in the immediate past has been identification of new chlamydiae and chlamydia-like bacteria and identification of broader host ranges. These remain key targets for future research. Ready availability of high-throughput DNA sequencing will facilitate research on distribution, hosts, and taxonomy and phylogenomics.

In addition, molecular approaches will accelerate the process of vaccine development. This is a vital area of endeavor, in that current vaccines are often lacking in efficacy and duration of immunity.

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31
Rickettsiales

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INTRODUCTION

Rickettsial pathogens cause several important and potentially fatal diseases of animals and humans, resulting in a significant health and economic burden. Rickettsiae represent some of the oldest yet most recently recognized pathogens of animals and man. Over the last few years, rickettsial diseases have emerged and reemerged in many parts of the world, probably in response to global warming, globalization, changes in lifestyle, and other drivers (Harrus and Baneth 2005).

Recently, organisms of the order Rickettsiales have been reclassified based on sequence analysis of their 16S rRNA, citrate synthase (*gltA*) gene, *groESL* operon gene, and their outer membrane protein (OMP) genes (Dasch and Weiss 1998; Dumler et al. 2001). The order Rickettsiales now includes two families, Anaplasmataceae and Rickettsiaceae, both of which include bacteria pathogenic for animals. The Anaplasmataceae constitutes the major family that is pathogenic to animals (table 31.1).

The Anaplasmataceae are distributed worldwide in endemic foci and are responsible for sporadic and seasonal outbreaks of disease. Some of these pathogens have the ability to cause disease in more than one host, and in this regard *Anaplasma phagocytophilum* is outstanding in its capacity to affect a wide range of mammals including man (Rikihisa 2006). Anaplasmataceae are all vector-borne parasites transmitted by ticks or trematodes. They are small gram-negative pleomorphic cocci that are obligate intracellular bacteria replicating in membrane-bound vacuoles in the cytoplasm of endothelial and hematopoietic cells (Walker et al. 2003). Infection may lead to clinically overt illness, and recovered animals often remain persistently infected, serving as a reservoir of the organism.

Of the members of the family Anaplasmataceae, the genomes of *A. phagocytophilum*, *Anaplasma marginale*, *Ehrlichia ruminantium*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Neorickettsia sennetsu* have been sequenced (Rikihisa 2006). These sequences are facilitating a better understanding of the pathogenesis of these important pathogens and may assist in the development of vaccines and more specific diagnostic methods.

This chapter will review current knowledge of the pathogenesis of the major rickettsial organisms causing disease in domestic animals.

FAMILY ANAPLASMATACEAE

Anaplasma marginale, *Anaplasma centrale*, and *Anaplasma ovis*

Background, Etiology, and Epidemiology

Anaplasmosis, commonly known as gall sickness, is an intraerythrocytic rickettsial infection of cattle, sheep, and wild ruminants. *A. marginale*, the most prevalent tick-borne pathogen of livestock, and the less virulent *Anaplasma centrale* invade and replicate in mature erythrocytes of cattle and wild ruminants, while *Anaplasma ovis* infects erythrocytes of sheep and goats. *Anaplasma* species can be distinguished microscopically by their location in the red blood cell, but *A. ovis* is microscopically indistinguishable from *A. marginale*. Anaplasmosis is

Table 31.1. Rickettsial Organisms Pathogenic for Domestic and Wild Animals

	Animal species affected	Disease
1. Family Anaplasmataceae		
Anaplasma spp.		
A. phagocytophilum	Ruminants	Tick-borne fever
	Equine	Equine granulocytic anaplasmosis
	Canine	Canine granulocytic anaplasmosis
	Feline	Feline granulocytic anaplasmosis
A. marginale	Bovine	Anaplasmosis
A. centrale	Bovine	Anaplasmosis
A. platys	Canine	Canine infectious cyclic thrombocytopenia
A. ovis	Ovine and caprine	Anaplasmosis
Ehrlichia spp.		
E. canis	Canine	Canine monocytic ehrlichiosis
E. chaffeensis	Canine	Canine monocytic ehrlichiosis
E. ewingii	Canine	Canine granulocytic ehrlichiosis
E. ovina	Ovine	Ovine ehrlichiosis
E. ruminantium	Bovine, ovine, caprine, and wild ruminants	Heartwater, cowdriosis
Neorickettsia spp.		
N. helminthoeca	Canine	Salmon poisoning
N. risticii	Horses	Potomac horse fever
	Canine	Atypical canine ehrlichiosis
2. Family Rickettsiaceae		
R. rickettsii	Canine	Rocky Mountain spotted fever

endemic worldwide in most tropical and subtropical areas, and its distribution depends on its tick vectors.

Phylogenetic studies based on the membrane surface protein (MSP) 1a have been used to characterize the genetic diversity of *A. marginale* worldwide (de la Fuente et al. 2007). Strains of *A. marginale* were found not to cluster according to geographic origin. Large numbers of repeat sequences were present in strains from a particular geographic area with a high degree of sequence variation within endemic areas. Genetic heterogeneity among strains of *A. marginale* could be explained by cattle movement and the infection of a single host with multiple *A. marginale* strains. Tick vector and tick pathogen interactions could also influence the presence of unique MSP1a repeats in strains of *A. marginale*.

Pathogenesis

Anaplasmosis is manifested by clinical signs of anemia, icterus without hemoglobinuria, fever, weakness, and depression. *A. marginale* causes considerable economic losses to the cattle industry and is the most pathogenic of the *Anaplasma* species listed above; most of the discussion of the pathogenesis of anaplasmosis centers about this organism. Although 20 species of ticks have been incriminated as vectors of *A. marginale*, ixodid ticks are the main vectors in cattle, and *Dermacentor* species including *Dermacentor variabilis*, *Dermacentor andersoni*, and *Dermacentor albipictus* are the major vectors in the United States (de la Fuente et al. 2001b). In South Africa, *Boophilus decoloratus* is the most important vector and four other tick species: *Rhipicephalus microplus*, *Rhipicephalus simus*, *Rhipicephalus eversti eversti*, and *Hyalomma marginatum rufipes* have been shown to transmit the infection experimentally. In Australia, only a single vector, *Rhipicephalus microplus*, has been recognized as the vector of bovine anaplasmosis. Among the ticks transmitting *A. ovis* are *Rhipicephalus bursa* and *D. andersoni*. The disease may also be transmitted mechanically by bloodsucking flies iatrogenically, orally, and by the intrauterine route.

Following initial acquisition feeding of a blood meal into the midgut lumen of the tick, the rickettsia

enters the midgut epithelial cells and undergoes the first round of replication. The bacterium then migrates to and invades the salivary glands. Apparently dependent on resumption of tick feeding on a mammalian host, a second round of replication takes place in the acinar cells followed by transmission in the saliva (Ueti et al. 2007). The incubation period of anaplasmosis caused by *A. marginale* lasts from 15 to 36 days. The disease manifests itself in a peracute, acute, chronic, or subclinical form. The intensity and duration of the disease is dependent on a number of factors: older animals are more prone to severe disease; animals on a reduced energy intake show less severe clinical disease; *Bos indicus* breeds appear to be more resistant than *Bos taurus* breeds.

The entry of *A. marginale* into the erythrocyte is facilitated by the MSP1a, an adhesin for bovine erythrocytes, and by increased erythrocyte membrane permeability resulting from a decrease in the acetylcholinesterase activity of the cell (Sharma and Tripathi 1985; McGarey and Allred 1994; de la Fuente et al. 2001a). The MSP1a protein is glycosylated, a characteristic which appears to contribute to its adhesive properties and thus the function of the protein (Garcia-Garcia et al. 2004).

The anemia is due to excessive erythrophagocytosis initiated by parasite-induced erythrocyte damage and the sensitizing effect of antierythrocytic autoantibodies on both infected and uninfected erythrocytes. The damaged or sensitized erythrocytes are removed by the monocyte-macrophage system. Other nonimmune factors that may play a role in the anemia are a factor stimulating nonspecific erythrophagocytosis, altered phosphofructokinase activity, and lowered levels of adenosine triphosphate (ATP) in the erythrocytes resulting in functional disturbances and changes in the protein and glycoprotein components of membranes of the infected erythrocytes.

The peracute form is rare but fatal and occurs most frequently in purebred animals. Animals suffering from this form die within a few hours. In the acute form, the mucous membranes are pale, and the animals are depressed, anorexic, and weak. Milk production drops and some animals develop fever. Icterus is evident later in the course of the disease. The chronic disease follows the acute disease and may persist for several months. This phase is characterized by anorexia, weight loss, anemia, and icterus.

Cattle that recover from *A. marginale* infection are persistently infected and are immune to reinfection. These animals remain carriers of *A. marginale* and may serve as reservoirs for tick transmission. Persistent infection is fundamental to the epidemiology of the disease, providing a reservoir for transmission during periods of peak arthropod activity. *A. marginale* persistent infection is characterized by sequential emergence, replication, and immune control of organisms expressing antigenic variant MSP2. Cyclic bacteremia persists for at least 6 years and appears to be lifelong in infected ruminants (Palmer et al. 2006). Variation in the MSP2 is generated by a process of gene conversion in which unique hypervariable region sequences (HVRs) located in pseudogenes are recombined into a single operon-linked *msp2* expression site. The complete genome sequence of the St. Maries strain of *A. marginale* has identified 7 HVR copies (Brayton et al. 2005). The new antigenic variants are not recognized by the antibody present at the time of their emergence and are controlled by the development of IgG2 antibodies directed against the specific HVR. The cycle of emergence and control continue unabated, allowing lifelong persistent *A. marginale* infection (Eriks et al. 1989). Thus, it appears that *A. marginale* is composed of a heterogenous bacterial population that evolves in the host or that the genotypic diversity implies a high transmission intensity by the vector, or both (Molad et al. 2008).

A. ovis is the main cause of ovine and caprine anaplasmosis and is more severe in goats than in sheep (Stoltsz 1994). Unlike the situation in bovine anaplasmosis, there appears to be no marked variation in age susceptibility. Overt clinical disease and mortality are rare. Sheep and goats that recover from *A. ovis* infection remain persistent carriers.

The ultimate vaccine for the control of anaplasmosis would be one that reduces infection by a wide range of isolates and the transmission of the pathogens by ticks (de la Fuente et al. 2006). To date, this has only been achieved with inoculations of live *A. centrale* in infected blood. Recombinant vaccines based on known MSPs have only induced partial protection, and conserved proteins may be necessary in developing a broad-range vaccine (Vega et al. 2007). Complete serological cross-reactivity has been demonstrated for *A. marginale* and *A. ovis*; however, infection with one does not provide protection against the other (Splitter et al. 1955, 1956). The *msp5* gene is highly conserved among all

Anaplasma species, and cross-reactivity has been demonstrated between the MSP5 of *A. marginale* and *A. phagocytophilum* (Strik et al. 2007).

Anaplasma phagocytophilum

Background, Etiology, and Epidemiology

Previously, *Ehrlichia phagocytophilum*, *Ehrlichia equi*, and the agent of human granulocytic ehrlichiosis (HGE) were considered as three distinct species. Recently, they have been reclassified as a single species named *A. phagocytophilum* (Dumler et al. 2001). Natural infection is transmitted transstadially by *Ixodes* ticks, mainly *Ixodes ricinus* and *Ixodes pacificus* which feed on a wide range of vertebrate animals, giving the organism the opportunity to cause disease in a wide variety of host species (Stuen 2007). The tick *Haemophysalis punctata* may also serve as a vector. Free-living rodents have been shown to harbor variants of *A. phagocytophilum*, suggesting that rodents may also be a reservoir of infection (Woldehiwet 2006). Migrating birds may also play an important role in the dispersal of *A. phagocytophilum*-infected *I. ricinus* in Europe (Skoracki et al. 2006).

A. phagocytophilum is the cause of tick-borne fever (TBF) also known as "pasture disease" in cattle, goats, sheep, and wild ruminants and of granulocytic anaplasmosis (previously ehrlichiosis) in horses, dogs, cats, and llamas. Infection has also been identified in European bison, wild boars, red foxes, donkey, small rodents, moose, and roe deer. Antibodies that react with *A. phagocytophilum* have been detected in hare and Euroasian lynx (Stuen 2007).

Pathogenesis

The natural incubation period of TBF is 3–13 days. Most outbreaks of TBF occur immediately after sheep and cattle have been introduced into tick-infested pastures. Isolated outbreaks have also been reported in goats. The onset of the disease is marked by a high fever of 1–2 weeks duration. In nonpregnant and nonlactating animals, the signs tend to be mild and nonspecific. Pregnant animals may abort. Clinical signs include reduced appetite, apathy, decreased milk production, and respiratory abnormalities. Death from uncomplicated disease is rare. An important consequence of *A. phagocytophilum* infection is exacerbation of concurrent infections, probably due to its immunosuppressive effects. The susceptibility to secondary infections may be the result of leukopenia and the organism's adverse effects on lymphocyte and neutrophil function. By infecting and actively growing in neutrophils, *A. phagocytophilum* is able to employ an array of mechanisms to subvert bactericidal activities which include inhibition of phagosome-lysosome fusion, suppression of respiratory burst, and the delay in apoptotic death of neutrophils (Woldehiwet 2008).

Following infection, there is a significant reduction in peripheral leukocytes, attributable to a profound and transient B cell lymphopenia, followed by an intense and enduring neutropenia and eosinopenia. A significant thrombocytopenia appears early in the course of the disease. A mild anemia has been demonstrated in experimental infection, and the hematologic changes in sheep are more severe than in goats (Gokce and Woldehiwet 1999). Rickettsemia was demonstrated 3 days post infection in sheep and goats, with about 70% of leukocytes displaying intracytoplasmic morulae. The rickettsemia declined after 8 days post infection and remained at low levels until about day 20 post infection. In cattle, morulae appear 3–4 days after infection. Peak rickettsemia appears on days 5–9 post infection, and morulae can be detected until about day 23 post infection (Brun-Hansen et al. 1998). Following experimental infection, *A. phagocytophilum* persists for up to 2 years (Woldehiwet 2006).

Pathological findings due to infection with *A. phagocytophilum* are similar for humans, horses, and sheep. These findings included splenic lymphoid depletion, small macrophage aggregates and apoptosis in the liver, and paracortical hyperplasia in the lymph nodes (Lepidi et al. 2000). Studies in mice suggest that histopathologic lesions may be due to immunopathologic mechanisms initiated by the organism. Interferon-γ (IFN-γ) plays an important role in the clearance of the organism in the early phases of infection, and may also play a role in the pathology. Interleukin (IL)-10 moderates the pathology, possibly through downregulatory effects on IFN-γ or through other anti-inflammatory mechanisms (Martin et al. 2001).

The mechanisms of intracytoplasmic survival and multiplication of *A. phagocytophilum* in polymorphonuclear leukocytes have been reviewed (Carlyon and Fikrig 2003). *A. phagocytophilum* inhibits phagosome-lysosome fusion due to an active bacterial process and not due to lysosomal dysfunction. Another means for *A. phagocytophilum* intracellular survival is through inhibition of reactive oxygen

species (ROS) by the cell. *A. phagocytophilum* lowers NADPH oxidase activity in neutrophils, downregulating the genes encoding protein components of NADPH oxidase and scavenging ROS by superoxide dismutase (Carlyon et al. 2004; IJdo and Mueller 2004).

Sheep that recover from TBF remain persistently infected with *A. phagocytophilum* and may serve as a reservoir for the organism from one grazing season to the next. Polymerase chain reaction (PCR) studies have shown that the detectability of *A. phagocytophilum* in artificially infected sheep declines sharply 3 months after infection (Stuen et al. 2006). These authors suggested that tick infestation and feeding may amplify *A. phagocytophilum* infections in carrier sheep, thus enabling tick transmission to occur. There is reduced weight gain due to subclinical infection with *A. phagocytophilum* in lambs, indicating that infection may contribute to productivity losses (Stuen et al. 2002). The mechanism by which *A. phagocytophilum* evades the host's immune system is probably similar to that described for *A. marginale*. In both pathogens, the major immunodominant surface antigen MSP2 (p44) are encoded by genes of a multigene family which persistently generate antigenic variants (Caspersen et al. 2002).

A. phagocytophilum infection in sheep is characterized by immune suppression. A reduction of percentages of γδ-T-cells and $CD4^+$ T cells in the peripheral blood with a downregulation of CD25 expression and a relative increase in $CD8^+$ T cells has been demonstrated in sheep vaccinated with commercial vaccines while infected with *A. phagocytophilum*. There was a reduction in expression of CD11b and CD14 on granulocytes and in the percentages of B cells and leukocytes expressing major histocompatibility complex class II (MHC-II) and CD11b. Antibody responses to vaccines, lymphocyte *in vitro* proliferative responses, and *in vitro* IFN-γ responses to antigens were reduced in sheep infected with *A. phagocytophilum* (Whist et al. 2003). Delayed neutrophil apoptosis has been demonstrated in sheep infected with *A. phagocytophilum*, possibly explaining how the bacteria replicate in these normally short-lived cells (Scaife et al. 2003). Infection also increases respiratory burst in neutrophils but decreases chemotaxis in these cells.

Equine granulocytic anaplasmosis is characterized by a mild, sometimes subclinical, and self-limiting disease. The clinical course of the disease ranges from 3 to 21 days, and when untreated can be self-limiting. Clinical signs include fever, anorexia, listlessness, depression, limb edema, petechiae, and icterus. Neurological signs include stiffness of gait, incoordination, ataxia, stumbling, unwillingness, and recumbency. Postmortem changes consistent with disseminated intravascular coagulation were noted in a horse that died following infection with a high intravenous dose of *A. phagocytophilum* (Franzen et al. 2007). Young horses may cough and loose weight. Hematological changes include early thrombocytopenia, leukopenia, neutropenia, lymphopenia, and mild anemia (Franzen et al. 2005). IL-1β and TNF-α were up-regulated in horses inoculated intravenously with *A. phagocytophilum* (Kim et al. 2002). These two cytokines are considered responsible for the *A. phagocytophilum*-induced mild fever, neutropenia, and thrombocytopenia. Morulae are present in the neutrophils of acutely ill horses shortly after the appearance of the first clinical signs. Seroconversion occurs in artificially infected horses 12–16 days post infection and 6–8 days after the onset of clinical symptoms (Franzen et al. 2005). PCR tests were positive 2–3 days before clinical signs and remained positive for 4–9 days after rectal temperature returned to normal.

Infection of dogs with *A. phagocytophilum* causes canine granulocytic anaplasmosis. Clinical signs vary in severity and include pyrexia, depression, anorexia, lymphadenopathy, polyarthritis, meningitis, and meningoencephalitis. In dogs experimentally infected with the Swedish strain of *A. phagocytophilum*, fever developed 4–7 days post infection (Egenvall et al. 1998). Leukopenia, lymphopenia, neutropenia, and moderate to severe thrombocytopenia were detected. Persistent infection with *A. phagocytophilum* has been reported to occur in dogs (Egenvall et al. 2000). Postmortem examination of dogs in the acute phase of the disease revealed an enlarged and reactive spleen and a nonspecific reactive hepatitis. Immune-mediated hemolytic anemia and thrombocytopenia caused by *A. phagocytophilum* has been described in a dog (Bexfield et al. 2005).

Feline granulocytic anaplasmosis has been described in several cats as a nonspecific clinical entity. Signs included lethargy, anorexia, fever, tachypnea, and dehydration. Cats infected with *A. phagocytophilum* developed antinuclear antibodies and increased levels of IFN-γ mRNA (Foley et al. 2003). Immunosuppression by feline immunodeficiency virus (FIV) in cats did not increase

the severity of anaplasmosis and may have attenuated immune responses to *A. phagocytophilum* infection (Foley et al. 2003). FIV-positive cats infected with *A. phagocytophilum* exhibited elevated IL-10 but not IFN-γ transcription. IL-10 reduces the duration of neutrophil apoptosis which could help the host eliminate the infection in neutrophils. In Sweden, the etiologic agent was visualized in neutrophils of a cat infested with *I. ricinus* ticks and found to show 100% identity with the DNA sequences of Swedish canine, equine, and human agents of granulocytic anaplasmosis (Bjoersdorff et al. 1999). *A. phagocytophilum* DNA has been detected in the blood of clinically ill domestic cats in the United States by use of PCR and DNA sequencing (Lappin et al. 2004). Serum samples were indirect immunofluorescence antibody (IFA) assay positive and antibody titers persisted for 21–30 days after treatment. Feline granulocytic anaplasmosis has been reported in the United Kingdom, Italy, and Spain.

An ehrlichial organism was identified in the blood of a sick llama with nonspecific symptoms that included partial anorexia, slight ataxia, and lethargy. Rare cytoplasmic inclusions were present. Buffy-coat cells were strongly positive by nested PCR for *A. phagocytophilum*. Following treatment with tetracyclines, the llama improved rapidly. The agent was also found in a pool of *I. pacificus* ticks collected in the region of the sick animal (Barlough et al. 1997).

Anaplasma platys

Background, Etiology, and Epidemiology

Anaplasma platys, formerly known as *Ehrlichia platys*, is the cause of infectious canine cyclic thrombocytopenia (ICCT) (Dumler et al. 2001). *A. platys* is the only known rickettsia to infect canine platelets.

ICCT has been reported in the United States, Israel, Greece, Japan, Thailand, Venezuela, China, Spain, Australia, and South Africa. *A. platys* is assumed to be transmitted by *Rhipicephalus sanguineus*, the brown dog tick, although experimental studies have failed to prove this mode of infection (Simpson et al. 1991). *A. platys* DNA has been detected in *Rhipicephalus sanguineus* ticks from free-roaming dogs in Japan and Africa.

Pathogenesis

ICCT occurs in subclinical and acute clinical forms. The latter form of the disease with distinct clinical signs has only been described in Greece and Israel and may be attributable to a more virulent strain of *A. platys* in the Mediterranean region (Kontos et al. 1991; Harrus et al. 1997). The clinical signs described in cases of natural infection include lymphadenomegaly, pale mucous membranes, and fever. The main hematologic and biochemical findings are thrombocytopenia and the presence of megaplatelets, anemia, monocytosis, and lowered albumin concentrations. Uveitis has been described in natural *A. platys* infection.

The incubation period after experimental intravenous infection is 8–15 days. *A. platys* infection causes thrombocytopenia, which occurs in cycles of approximately 10–14-day intervals. The severity of the thrombocytopenia and the percentage of platelets that are parasitized are maximal during the first parasitemic phase. The degree of thrombocytopenia, however, does not seem to be dependent on the rate of parasitemia and the thrombocytopenia may be severe in cases with a low parasitemia. It is proposed that whereas the thrombocytopenia in the initial parasitemic phase may be due to injury by the replicating parasites, immune-mediated mechanisms may be important in subsequent thrombocytopenic episodes.

Dogs experimentally infected with *A. platys* develop a mild normocytic normochromic anemia (Baker et al. 1988). The changes in erythrocytes and serum iron concentrations were similar to those described for dogs with anemia of inflammation. There was a significant increase in alanine aminotransferase and alkaline phosphatase 7 days post infection, and these enzymes remained elevated for up to 35 days post infection.

Postmortem gross findings are limited to generalized lymphadenomegaly. Microscopically, the lymph nodes show follicular hyperplasia with mild necrosis of follicular lymphocytes, which is more evident later in the infection. Plasmacytosis is evident 2 weeks post infection. Follicular hyperplasia occurs in the spleens of artificially infected dogs. Crescent-shaped splenic hemorrhages are seen in dogs 14 days post infection. The liver contains multifocal lymphocytic accumulations and the kidneys mild multifocal lymphocytic and plasmacytic infiltrations. Bone marrow cellularity is increased (Baker et al. 1987).

The first antibody titers against *A. platys* are detectable coincidentally with the initial parasitemia. There is no serological cross-reaction between *E.*

canis and *A. platys* (Waner et al. 2001). In an artificial infection study in dogs using real-time PCR detection of *A. platys* in blood and tissues, buffy-coats were PCR positive 4 days after inoculation and remained positive in all dogs until day 14, during which time marked thrombocytopenia and low parasitemia were observed. Between 17 and 28 days post inoculation, the PCR results of buffy-coats were intermittently negative and there was no microscopic evidence of parasitemia. Bone marrow and splenic aspirates collected from 50% of the infected dogs were positive by real-time PCR at 28 days post inoculation, when PCR results for buffy-coats were negative. The results indicate that spleen and/or bone marrow samples should be considered as additional samples for PCR testing of dogs during the acute phase of *A. platys* infection (Eddlestone et al. 2007).

Coinfection with other tick-borne diseases may confuse the clinical pathological picture and should always be considered (Loftis et al. 2006; Manzillo et al. 2006; Heyman et al. 2007). There is a multiplex-molecular real-time PCR assay that can detect coinfections with *Ehrlichia* and *Anaplasma* species in dogs (Sirigireddy et al. 2006).

Ehrlichia canis

Background, Etiology, and Epidemiology

Ehrlichia canis, the etiologic agent of canine monocytic ehrlichiosis (CME), parasitizes circulating monocytes intracytoplasmically in clusters of organisms called morulae. This organism has been recognized worldwide as an important infectious agent of dogs and other canids. Human patients in Venezuela with clinical signs compatible with human monocytic ehrlichiosis (HME) were infected by a closely related organism which was suggested to be a strain of *E. canis* (Perez et al. 2006).

E. canis is transmitted by *Rhipicephalus sanguineus*; the mode of transmission is transstadial, but not transovarial (Smith et al. 1976). Larvae and nymphs become infected while feeding on rickettsemic dogs and transmit the rickettsia to the host after moulting to nymphs and adults, respectively. While feeding, ticks inject *E. canis*-contaminated saliva into the feeding site. Adult ticks transmit the infection for at least 155 days after becoming infected. This allows ticks to overwinter and infect susceptible dogs in the following spring (Lewis et al. 1977). Most CME cases occur during the warm season when the vector ticks are abundant. Infection with *E. canis* may also occur through infected blood transfusions.

Pathogenesis

The genome of *E. canis* has recently been sequenced and was found to consist of a single circular chromosome of 1,315,030 nucleotides. Significant findings include its small size relative to other ehrlichiae and the presence of a similar number of predicted genes. A total of 984 genes were identified in the *E. canis* genome. In accordance with the organism's intracellular existence, the genome has undergone severe loss of metabolic pathway enzymes (Mavromatis et al. 2006).

Immunoreactive surface proteins suspected to be adhesins have been identified possessing repeat units with high serine and threonine content. Based on the sequence similarity with proteins from *E. chaffeensis* and *E. ruminantium*, it has been suggested that they are involved in the ehrlichial attachment and entry into the host cell (Popov et al. 2000; Yu et al. 2000). The incubation period of CME is 8–20 days. The organisms multiply in macrophages of the monocytic phagocytic system, by binary fission, spreading throughout the body. Replication in the host takes place in secluded membrane-bound vacuoles protected from the host immune surveillance system, lysosomes, and oxygen reactive intermediates. Ankyrin genes encoding proteins have been identified and are suggested to provide a mechanism for adaptation that allows ehrlichiae to reside within vacuoles and communicate with the host cell through the endoplasmic reticulum mediating specific protein–protein interactions (Mavromatis et al. 2006). Ankyrin proteins affect proinflammatory cytokine expression and the downregulation of cell cycle regulators.

E. canis, like other *Ehrlichia* and *Anaplasma* spp., lacks enzymes for the biosynthesis of peptidoglycans and lipopolysaccharide (LPS) that provide strength to the outer membrane (Lin and Rikihisa 2003). It has been proposed that *Ehrlichia* and *Anaplasma* spp. have become dependent on their hosts cholesterol, as depletion of membrane cholesterol or alteration in its structure disrupts their structural integrity, rendering the organism unable to infect their host cells. The dependence of *Ehrlichia* and *Anaplasma* spp. on cholesterol may have clinical consequences, an issue that requires further investigation. The absence of LPS and peptidoglycans also has important implications for the infection and survival of *E. canis* in both ticks and dogs. The tick's immune system responds to the presence of LPS, and the absence of LPS

in ehrlichial organisms gives them a survival advantage. In the mammalian host, macrophages or neutrophils use pattern recognition receptors, such as Toll-like receptors (TLRs), that bind to molecules with conserved pathogen-associated molecular patterns (PAMPs), such as LPS or peptidoglycan, eliciting intense innate immune responses intended to eliminate the invading microorganisms. The absence of LPS and peptidoglycan gives *E. canis* an added advantage in intraleukocyte survival.

Persistent infection with *E. canis* has been documented in dogs for a period of 3 years (Harrus et al. 1998b). Evasion of the host immune system is through constant alterations of the surface architecture and/or the expression of different protein variants. In this regard, proteins with tandem repeats play an important role in the pathogenicity and pathogen–host cell interaction. Twelve proteins containing tandem repeats have been identified in the *E. canis* genome, and with the exception of three proteins that appear specific to *E. canis,* all the others have orthologs in other genomes from Rickettsiales (Mavromatis et al. 2006). Persistent immune evasion may also involve downregulation of MHC class II receptors (Harrus et al. 2003). This probably leads to impairment of signalling pathways affecting antigen presentation, cell–cell adhesion, cytokine production, humoral reactions, isotype switching, and antirickettsial activity.

The incubation period is followed by three consecutive stages: acute, subclinical, and chronic. The acute phase may last 1–4 weeks, during which signs such as fever, oculonasal discharge, anorexia, depression, petechiae, ecchymoses, lymphadenomegaly, and splenomegaly may occur. Thrombocytopenia is most prevalent during the acute stage. Mild leukopenia and anemia may also occur. Most dogs recover from the acute disease with adequate treatment. Untreated dogs and those treated inappropriately may enter the subclinical phase, in which there are no clinical signs but platelet counts may be subnormal (Waner et al. 1997; Harrus et al. 1998b). Dogs in this phase may remain persistent carriers of *E. canis* for months and even years (Harrus et al. 1998b). Persistently infected dogs may recover from the disease spontaneously, or may develop chronic severe disease. Not all dogs develop the chronic phase of CME, and the conditions leading to the development of this phase remain unclear. Dogs suffering from the chronic phase may develop clinical signs similar to those of the acute disease but with greater severity. Severe pancytopenia is a typical finding of the chronic disease, which occurs as a result of hypocellular bone marrow. Significantly lower white blood cell, red blood cell, and platelet counts were found to be a high risk for mortality. Severe leucopenia severe anemia, prolonged activated partial thromboplastin time, and hypokalemia have each found to predict mortality with a probability of 100% (Shipov et al. 2008). Death may occur as a consequence of hemorrhages and/or secondary infections.

Gross pathological lesions in affected dogs include hemorrhages (petechiae and ecchymoses) in subcutaneous tissues and on serosal and mucosal surfaces of most organs. Generalized lymphadenomegaly and splenomegaly are common findings. *E. canis* DNA has been detected at necropy of naturally infected dogs in a variety of tissues (Gal et al. 2008).

Histopathologic findings include lymphocytic, plasmacytic, and monocytic infiltration, and perivascular cuffing in numerous organs, mainly the lungs, brain, meninges, kidneys, lymph nodes, bone marrow, and spleen. The bone marrow is hypercellular and red in the acute phase but hypocellular, fatty, and pale in the chronic disease (Hildebrandt et al. 1973; Neer 1998). Ocular and central nervous system findings are common. Nonsuppurative meningitis or meningoencephalitis may occur. The meningitis in CME is often accompanied by mild neuroparenchymal vascular cuffing and gliosis. Ocular signs involve most structures of the eye and include conjunctivitis, conjunctival or iridal petechiae and ecchymoses, corneal edema, uveitis, and hyphema (Hildebrandt et al. 1973; Panciera et al. 2001). In a recent study of natural CME, anterior bilateral uveitis appeared to be the most prevalent ocular lesion, with a favorable outcome with systemic and topical treatment in the majority of the affected dogs (Komnenou et al. 2007).

Most dogs infected with *E. canis* develop hyperproteinemia due to hypergammaglobulinemia, which is usually polyclonal but may be monoclonal in some dogs (Harrus et al. 1999). The latter dogs may develop hyperviscosity with associated pathology and clinical signs. Cases of sudden blindness due to subretinal hemorrhage associated with hyperviscosity have been documented (Hoskins et al. 1983; Harrus et al. 1998a).

As a multisystemic disease, ehrlichiosis has the potential to cause disease in a variety of organ

systems. Evidence for ehrlichiosis as a risk factor for myocardial injury has been suggested by increased levels of cardiac tropinin 1 in dogs with acute *E. canis* (Diniz et al. 2008) infection and in a case where apparent electrocardiographic changes were detected associated with infection (Waner and Ohad 2008).

Although immunologic responses do not appear to be grossly impaired in young dogs during the first few months after experimental *E. canis* infection (Hess et al. 2006), immunologic mechanisms appear to be involved in the pathogenesis of the disease by the induction of antiplatelet and/or antierythrocyte antibodies and immune complexes (Harrus et al. 1996b, 2001a). Platelet-bindable and platelet-bound antiplatelet antibodies were demonstrated post infection and are suggested to play a role in the pathogenesis of thrombocytopenia in CME (Harrus et al. 1996b; Waner et al. 2000). Other mechanisms involved in the development of the thrombocytopenia during the acute phase include increased platelet consumption, splenic sequestration, shortened platelet life span, and the presence of a platelet migration-inhibition factor (Abeygunawardena et al. 1990; Harrus et al. 1999).

There is platelet dysfunction in infected dogs. It was suggested that antiplatelet antibodies are involved in the thrombocytopathy in CME by competitive binding to the platelet receptors (Harrus et al. 1996a). The greater the magnitude of the thrombocytopenia, the greater the possibility for detection of *E. canis* 16S rRNA in the blood of dogs.

Following experimental infection, IgG antibodies generally appear 15 days post infection (Waner et al. 2001). IgG2 antibody reaction to *E. canis* is the predominant response in the various phases of the disease (Harrus et al. 2001b; McBride et al. 2003). It has been proposed that the isotype switching to IgG2 subclass antibodies in dogs is associated with a T-helper type 1 (TH-1) response and a corresponding production of IFN-γ (McBride et al. 2003). This suggestion has been strengthened by the finding of persistent expression of IFN-γ and TNF-α mRNA from days 2–8 after infection of dogs with the Oklahoma strain of *E. canis* and continuing to day 56 post inoculation (Tajima and Rikihisa 2005). Furthermore, IFN-γ and TNF-α exert an antirickettsial effect via the induction of nitric oxide synthesis (Feng and Walker 2004).

IgM antibody response is not significant and may not be observed at all in the acute phase of the disease (McBride et al. 2003). The role of the humoral immune response in *E. canis*-infections is unclear. High *E. canis*-antibody titers do not provide protection for animals that are challenged and may have a detrimental effect on the disease outcome (Ristic and Hollland 1993; Breitschwerdt et al. 1998a). German shepherd dogs were more susceptible to CME than were other breeds and had more severe disease with a higher mortality rate. The cellular immune response of German shepherd dogs was depressed compared with that of beagle dogs, while no significant differences were recorded in the humoral response between the two breeds (Nyindo et al. 1980). This finding supports the predominant role of cell-mediated immunity during *E. canis* infection.

The indirect immunofluorescence antibody (IFA) assay is the "gold standard" for exposure to CME (Waner et al. 2001) but it has limitations such as cross-reactivity with related organisms, subjectivity, technical expertise required, and the high cost of the equipment. An early diagnosis of *E. canis* infection in the acute phase of the disease allows for early treatment and an excellent prognosis. Immunodiagnostics provide sensitive and specific tests, especially during the early acute phase of the infection. The 36- and 19-kDa proteins are the first antigens to elicit antibody response in *E. canis*-infected dogs (McBride et al. 2003), whereas antibodies to the 28- and 200-kDa proteins develop in the late-acute-phase immune response.

The dominant antibody epitopes of the major immunoreactive proteins of *E. canis* are serine rich tandem repeats which appear to be the primary targets of the humoral immune response. The predominance of antibody response to the acidic ehrlichial proteins is unique to the ehrlichia–host interaction directing the immune response (Nethery et al. 2007). Glycoprotein 200 (gp200), the largest major immunoreactive ehrlichial glycoprotein ortholog, contains ankyrin which has been proposed as a virulence factor due to its ability to facilitate intracellular infection (Lin et al. 2007). It has been proposed that antibodies inhibiting gp200 may benefit the host during infection (Nethery et al. 2007). *E. canis* organisms from different parts of the world are antigenically diverse, and it is possible that the severity of the disease may be affected by the strain of the organism (Hegarty et al. 1997).

Quantitative real-time PCR was used in order to evaluate *E. canis* infection in naturally and experimentally infected dogs. All experiementally infected dogs were positive for *E. canis* DNA 7 days post infection and developed clinical sings by 9–12 days post infection. A rapid increase in ehrlichial DNA was found to correlate with the appearance of severe clinical signs of disease. The mean spleen and blood DNA copies of *E. canis* significantly increased by more than 10-fold from 7 days post infection to 10–12 days post infection (Baneth et al. 2009).

Ehrlichia chaffeensis

Background, Etiology, and Epidemiology

Ehrlichia chaffeensis is the etiologic agent of HME (Dumler et al. 2001). The first diagnosis of an HME case occurred in 1986, 2 weeks after a tick bite in Arkansas (Maeda et al. 1987). Initially, the etiology was erroneously attributed to *E. canis* due to its appearance within monocytes and a serologic cross-reaction with *E. canis*. However, in 1990, *E. chaffeensis* was isolated from a patient at Fort Chaffee, Arkansas, and recognized as the cause of HME (Dawson et al. 1991). Since then, nearly 40 isolates have been obtained from humans and wild mammals (Varela-Stokes et al. 2006); however, it is unknown whether the different genetic variants cause different clinical manifestations in patients. “Infection exclusion,” a phenomenon whereby infection with one genotype excludes infection with another genotype, has not been observed in *E. chaffeensis* infections in white-tailed deer; the deer react to the major proteins of all strains to which they are exposed (Varela-Stokes et al. 2006).

E. chaffeensis has been identified from humans, deer, dogs, and ticks in the United States, mainly the south central, southeastern and Mid-Atlantic States, and California. Detection of *E. chaffeensis* DNA by PCR amplification has provided evidence for natural canine *E. chaffeensis* infection in southeastern Virginia, Oklahoma, and North Carolina (Dumler and Walker 2001).

E. chaffeensis is transmitted by *Amblyomma americanum* (lone star tick) and, to a lesser extent, by *D. variabilis* (American dog tick). Persistently infected white-tailed deer, and possibly canines, serve as reservoirs (Dumler and Walker 2001). In Cameroon, *Rhipicephalus sanguineus* also harbors *E. chaffeensis*, although its potential to be a competent vector is unknown (Ndip et al. 2007). Other tick species which may serve as vectors and or reservoirs of *E. chaffeensis* include *Haemophysalis longicornis* (Korea) and *Amblyomma testudinariun* (China).

Pathogenesis

The clinical significance of natural canine infection with *E. chaffeensis* has yet to be determined. The effects of subcutaneous experimental infection in beagle dogs were studied over a period of 6 months (Zhang et al. 2003). With the exception of thrombocytopenia, which became evident 2 weeks after infection and persisted for the duration of the study, no other clinical signs were detected. Antibodies were detected in the fourth week post inoculation and persisted for the duration of the study. In another study of pups experimentally infected with *E. chaffeensis*, fever was noted without any other signs (Dawson and Ewing 1992). One report associated vomiting, epistaxis, lymphadenomegaly, and uveitis in three dogs with *E. chaffeensis* infection (Breitschwerdt et al. 1998b).

Two forms of *E. chaffeensis* have been distinguished by electron microscopy: dense-cored (DC) and reticulate (RC) forms residing within the intracellular morulae (Popov et al. 1995; Zhang et al. 2007). Both forms divide by binary fission. The DC attaches and enters the host cell, whereas the RC represents the multiplication form. Analysis of the OMP expression has shown that RC expresses the 28-kDa OMP, the intermediate form expresses both gp120 and gp28, and the mature DC form only gp120. *E. chaffeensis* forms an abundant intramorular fibrillar matrix that appears to contain the gp120 shed from the surface of the DCs. The membrane of the morula does not contain gp120 (Popov et al. 2000). The gp120 protein may be an adhesin that enhances the internalization of the ehrlichiae.

E. chaffeensis, as an obligatory intracellular bacterium, lacks many genes involved in metabolism and must therefore take up various nutrients and metabolic compounds from its immediate cytoplasmic environment. This phenomenon occurs primarily through pores or channels in the bacterial outer membrane and has been identified in the major OMP of *A. phagocytophilum*, P44 (Huang et al. 2007). OMPs P28 and OMP-1 have now been suggested to act as porins in *E. chaffeensis* (Kumagai et al. 2008). It has been proposed that these proteins may serve as vaccine candidates.

E. chaffeensis resides primarily in monocytes and macrophages, and granulomas have been observed in tissues of HME patients who subse-

quently recovered from the illness. *E. chaffeensis* causes cytopathic effects and necrosis of heavily infected cells *in vitro* and necrosis in immunocompromised patients. Pathological lesions in humans include focal necrosis of the liver, spleen, and lymph nodes, multiorgan perivascular lymphohistiocytic infiltrates, hemophagocytosis in the spleen, liver, bone marrow, and lymph nodes, interstitial pneumonitis, and pulmonary hemorrhage (Brouqui and Dumler 1997). Secondary infections by opportunistic fungal and viral organisms have been documented in severe and fatal cases, and suggest the possibility of suppression or dysregulation of the immune response by *E. chaffeensis* (Brouqui and Dumler 1997).

Iron is essential for the maintenance of ehrlichial organisms, and *E. chaffeensis* up-regulates the host cell transferrin receptor (TfR) mRNA causing accumulation of iron in the morula membrane. IFN-γ-activated human monocytes inhibited *E. chaffeensis* infection by downregulation of surface TfRs, which led to the limitation of available cytoplasmic iron (Barnewall and Rikihisa 1994). This effect of IFN-γ indicates the importance of cell-mediated immunity in *E. chaffeensis* infections. Studies carried out using MHC-II knockout (KO) mice demonstrated persistence of infection indicating that cell-mediated immunity is critical for resistance to *E. chaffeensis* (Ganta et al. 2002, 2004). However, OMP-specific monoclonal antibodies protected severe combined immunodeficiency (SCID) mice from fatal infection with *E. chaffeensis*. This may indicate that antibodies, in the absence of cell-mediated immunity, can play a significant role in host defense during infection by this organism (Li et al. 2001).

E. chaffeensis may cause persistent infection despite active host immune response (Telford and Dawson 1996). *E. chaffeensis* was cultured and DNA was detected in the blood of two experimentally infected dogs 74–102 days and 81–117 days, respectively, after inoculation (Zhang et al. 2003). The results indicated that dogs might become carriers of *E. chaffeensis* and serve as a natural host of the organism without exhibiting clinical signs.

The multigene locus encoding 28-kDa surface antigen proteins of two *E. chaffeensis* isolates has been characterized. Variant forms of the 28-kDa (P28 OMP) were identified and extensive restriction fragment length polymorphism was noted among the isolates. It was suggested that short-term evolutionary changes such as genetic recombinations leading to antigenic variants allow immune evasion by the organism and therefore persistent infection (Reddy and Streck 1999; Singu et al. 2005). Antibodies to P28 OMP were detected from about 30 days post inoculation in dogs artificially infected with *E. chaffeensis* (Zhang et al. 2004).

E. chaffeensis also possesses a unique mechanism to overcome a bactericidal activity within the host cell by inactivating the mechanisms for generation of ROS (Lin and Rikihisa 2007). This is accomplished by the destabilization and degradation of $p22^{phox}$, a component of NADPH oxidase.

Ehrlichia ewingii

Background Etiology, and Epidemiology

Ehrlichia ewingii has been phylogenetically clustered with *E. canis*, *E. chaffeensis*, *Ehrlichia muris*, and *E. ruminatium* (Dumler et al. 2001). In this group, *E. ewingii* is the exception in that it is detected most frequently in peripheral blood neutrophils and it has not been grown in culture. In man, the disease has been recently named "human ewingii ehrlichiosis" (Dumler et al. 2007)

E. ewingii has been identified in a large variety of ticks including *Rhipicephalus sanguineus*, *A. americanum*, *D. variabilis*, *Ixodes scapularis*, and *I. pacificus*. Experimentally, only *A. americanum* has been shown to be a competent vector; however, natural infection of two other tick species, *Rhipicephalus sanguineus* and *D. variabilis*, has been reported in the United States (Murphy et al. 1998). *E. ewingii* is prevalent in dogs and ticks in Cameroon, and *Rhipicephalus sanguineus* has been suggested to be the natural agent of transmission (Ndip et al. 2005, 2007).

A. americanum tick and the white-tailed deer are important vector and reservoir hosts, respectively, for *E. ewingii* (Yabsley et al. 2002; Varela-Stokes 2007). The white-tail deer serves as a host for all stages of lone star ticks, and it has been proposed that the striking increase in the population of the white-tail deer in the United States has dramatically affected the frequency and distribution of *A. americanum*-associated diseases. The possibility that asymptomatic dogs may also serve as reservoirs has also been proposed (Liddell et al. 2003).

Pathogenesis

Fever and lameness are the most common clinical findings in dogs naturally infected with *E. ewingii*.

Other clinical signs include lethargy, anorexia, and vomiting. Neutrophilic polyarthritis has been identified as a cause of lameness in some infected dogs. Neurological abnormalities have been observed including ataxia, paresis, proprioceptive deficits, anisocoria, intention tremor, and head tilt. The predominant hematologic abnormality is thrombocytopenia. Other hematologic changes include anemia, leukocytosis, lymphopenia, monocytosis, and the presence of reactive lymphocytes and toxic neutrophils. Morulae consistent with *E. ewingii* infection can be identified in neutrophils in some dogs. In one study, morulae could be identified in the neutrophils on blood smears in 54% of dogs (Goodman et al. 2003). Subclinical infection has also been described. Coinfections with other tick-borne agents are frequent and may confound the clinical picture.

In a study investigating the effect of *E. ewingii* infection on dog neutrophils, it was found that infection significantly delayed neutrophil apoptosis. This appeared to be the result of stabilization of mitochondria by augmenting the integrity of the mitrochondiral membranes, thus delaying spontaneous apoptosis (Xiong et al. 2008).

Ehrlichia ruminantium

Background, Etiology, and Epidemiology

Ehrlichia ruminantium, previously known as *Cowdria ruminantium* (Dumler and Walker 2001), is the causative agent of heartwater or cowdriosis, an acute lethal infection of domestic and wild ruminants transmitted by *Amblyomma* ticks. At least 12 *Amblyomma* tick species are able to transmit *E. ruminantium*, although at different vector efficiencies (Camus et al. 1996). The most important and widespread are *Amblyomma hebraeum* and *Amblyomma variegatum* and their distribution is primarily in sub-Saharan Africa, where heartwater is a serious problem to livestock farmers. Heartwater also occurs on three Caribbean islands (Camus et al. 1996). The disease causes severe economic losses through high mortality, with rates of up to 90% in susceptible hosts (Mahan et al. 2001).

Heartwater occurs all year round where seasons are not distinct, as in eastern Africa and the Caribbean, or during the rainy season as observed in southern Africa. The distribution of the *Amblyomma* ticks determines the distribution of the disease. *Amblyomma* ticks acquire infection by feeding on clinically infected or carrier hosts. Transmission may also occur vertically or via the colostrum. *Amblyomma* ticks are important reservoirs because they can harbor *E. ruminantium* organisms for up to 15 months (Camus et al. 1996). The abundance of infection (clinical or carrier) in an animal population and the level of tick control influence the infection intensity in the ticks and its epidemiology.

The severity of disease due to *E. ruminantium* in domestic ruminants varies based on age and breed of the animal and virulence of the infecting isolate. Newborn lambs and kids possess an innate immunity during the first 4 weeks of life, and in calves this immunity lasts up to 6 months. If there is no exposure to *E. ruminantium* during this period, these animals become fully susceptible to infection (Du Plessis 1970). Sheep and goats are more susceptible than cattle, and European breeds are more susceptible to infection than indigenous African breeds (Camus et al. 1996). Several wildlife species are also susceptible to disease caused by *E. ruminantium*, but the majority remain refractory and serve as reservoirs of infection (Peter et al. 2002).

Pathogenesis

The pathogenesis of *E. ruminantium* is related to its predilection for macrophages, neutrophils, and endothelial cells. *E. ruminantium* multiplies by binary fission and forms colonies inside a cytoplasmic vacuole in host cells. *In vitro*, three morphological stages of the organism are recognized: the reticulate bodies, intermediate bodies, and elementary bodies. Binary fission occurs in the first two stages, but only the elementary bodies are infective (Camus et al. 1996). The macrophage is believed to be the first host cell to be infected by *E. ruminantium*. Infected macrophages drain into the regional lymph node, and the infection subsequently enters the blood circulation via the lymph and disseminates systemically, invading endothelial cells in the process.

Endothelial cell invasion is a major factor in *E. ruminantium* disease. Although the mechanisms of invasion are not known, it is expected that entry into phagocytes is facilitated by phagocytosis, whereas certain OMPs of the organism facilitate establishment of intracellular infection by adhesion and invasion of nonphagocytic cells. *E. ruminantium* undergoes cycles of invasion of host cells, multiplication, expansion into colonies, and release from cells by cellular disruption, and probably by cell-to-cell transfer via adherens junctions. This facilitates

dissemination and pathology in a variety of organs. Following the tick transmission of infection, an incubation period of 10–21 days (average of 18 days) ensues and nonspecific clinical signs of the disease commence. Fever (>41°C) is followed by clinical disease that is peracute, acute, subacute, or mild.

Sudden death is a common sequel to the peracute form, which lasts less than 2 days without any clinical signs. The acute form is most commonly observed, and a fever of 41–42°C is accompanied by nonspecific signs such as dyspnea, tachycardia, anorexia, coughing, oculonasal discharge, and dullness, followed by death. This form lasts for 2–6 days. Neurological signs may develop, which include leg paddling, muscle tremors, circling, hyperesthesia, twitching of eyes, head butting, and recumbency, followed by death. Diarrhea may be observed in terminal cases, especially in cattle. The subacute form lasts 7–10 days, is clinically similar to the acute form, except that the disease process is prolonged, and there is a higher recovery rate. Recovery is common after a 2- to 3-day febrile period in the mild form, which usually goes unnoticed. Long-term immunity develops in recovered animals unless the infecting *E. ruminantium* isolate is not cross-protective.

Clinical and General Pathology

Clinical pathological changes of *E. ruminantium* infection are associated with the acute febrile phase of the disease, when rickettsemia is at its highest and the organisms are readily isolated by culture. *E. ruminantium* infection causes progressive anemia (normocytic, normochromic), which is thought to be associated with bone marrow depression. The total and differential white cell counts fluctuate and are characterized by neutropenia, eosinopenia, and marked lymphocytosis, usually at the end of the clinical phase (Van Amstel et al. 1988, 1994). Thrombocytopenia, increased prothrombin, and activated partial thromboplastin time and fibrinogen are also evident. A decline in total calcium levels, total serum protein, and levels of albumin and globulin may also occur during the acute phase of the disease.

At post mortem, typical signs of *E. ruminantium* infection are hydropericardium (hence the term “heartwater”), hemorrhages on epicardium, endocardium, and on visceral and mucosal surfaces, hydrothorax, lung edema, ascites, edema of the mesenteric and mediastinal lymph nodes, and splenomegaly. The amount of fluid in the pericardial sac and/or thoracic cavity varies between 50 and 1000 ml. The lungs and kidneys may be congested and enlarged. Brain congestion and edema is sometimes observed and is thought to be associated with the neurological signs seen in the terminal stages of the disease. Effusion of fluids into the body cavities is due to seepage of plasma proteins, which is thought to occur due to vasculitis and increased permeability of blood capillaries. Death is a result of pulmonary edema, cardiac insufficiency, edema of the brain, and shock due to circulatory collapse.

Several hypotheses have been proposed for the observed increased vascular permeability, and include the direct destruction of endothelial cells by *E. ruminantium,* involvement of pathogen associated toxins, release of vasoactive amines, or pathology mediated by inflammatory cytokines that are released in response to infection. Microscopically, the most definitive finding and diagnosis of infection is the presence of *E. ruminantium* in brain capillary endothelium (Purchase 1945). The organisms occur in colonies in the cytoplasm or free in the capillary lumen in material taken from any part of the brain, although the hippocampus is the preferred choice as a diagnostic sample. Perivascular infiltrates of macrophages and lymphocytes are also seen in brain tissue from infected animals. Renal nephrosis is common, and *E. ruminantium* can also be found in the glomerular capillaries (Camus et al. 1996).

The pathophysiological mechanisms of hydropericardium, hydrothorax, and hemorrhages on serosal and mucosal surfaces are unclear. Based on observations from other endothelial cell-tropic rickettsiae, a number of pathogenic pathways during *E. ruminantium* infection may be predicted. One pathway leading to increased vascular permeability is the disruption of adherens cell–cell junctions of endothelial cells (Walker 2007). This permeability is further enhanced by production of ROS. Furthermore, the infected endothelial cells have the capacity to present pathogen antigens via MHC class I and II molecules and may provide a focus for infiltration by macrophages and T cells leading to vasculitis. This is observed in the spotted fever group (SFG) rickettsioses which infect endothelial cells (Damas et al. 2006). The role of CD40L ligands produced by activated platelets is another mechanism by which inflammation may lead to

vasculitis. Platelets secrete this molecule, which is a costimulatory ligand for the CD40 receptors on B cells, T cells, monocytes/macrophages, and endothelial cells, and hence promote inflammation. In SFG infections, there is an increase in the production of prostaglandins which promote inflammatory pathways (Rydkina et al. 2006). *E. ruminantium* infection of bovine endothelial cells *in vitro* elicits the *de novo* synthesis of proinflammatory cytokines IL-1β, IL-6, and up-regulation of IL-8 mRNA. Although IL-1 and IL-6 can act as costimulators of T and B cells, uncontrolled production of these proinflammatory cytokines and other mediators can contribute to the pathogenesis of *E. ruminantium* infection.

Immunology of Ehrlichia ruminantium *Infection*

Immunity to *E. ruminantium* can be acquired passively via colostrum by calves born to immune dams, or is acquired following recovery from infection. Protective immunity is cell mediated, involving cells of the TH-1 pathway. Recovery is usually followed by a persistently infected state. During primary *E. ruminantium* infection, an increase in $CD4^+$ T-cells, $CD8^+$ T-cells, and IgM and IgG surface positive B lymphocytes occurs in the peripheral blood. A polyclonal antibody response does not develop until after the febrile reaction. This response recognizes several immunodominant antigens including the major antigenic protein (MAP)-1 (a 28–30-kDa surface exposed protein; Jongejan and Thielemans [1989]; Barbet et al. [1994]), MAP-2 (21-kDa protein; Mahan et al. [1994]), 18HW (27-kDa analogous to the OMP of *Coxiella burnetti*; Barbet et al. [2001]), heat-shock protein (58-kDa antigen; Lally et al. [1995]), and other proteins (Barbet et al. 2001). These immunogenic proteins appear to be highly conserved between *E. ruminantium* strains. However, humoral immunity is unlikely to provide protection against *E. ruminantium* because of its intracellular location, which is inaccessible to antibodies. This has been demonstrated by the failure to transfer immunity by hyperimmune serum (DuPlessis and Malan 1987).

Activation of T lymphocytes of the TH-1 pathway ($CD4^+$ and/or $CD8^+$ T cells and IFN-γ-mediated pathways) is key to the induction and maintenance of protective immunity to *E. ruminantium* infection (Mwangi et al. 1998, 2002; Byrom et al. 2000a, 2000b). Splenocytes from immune mice secrete IFN-γ, IL-6, and low levels of IL-2 when stimulated with *E. ruminantium* antigens and recombinant MAP-1 and MAP-2. IFN-γ is produced when a classical TH-1-type immune response is activated involving $CD4^+$ and or $CD8^+$ T cells (Mosmann and Coffman 1989).

Peripheral blood mononuclear cells of immune cattle respond to autologous *E. ruminantium*-infected monocytes and endothelial cells, and to stimulation with MAP-1 and MAP-2 antigens. $CD4^+$ and γδ-T lymphocyte cell lines generated from these cultures secrete IFN-γ, and contain mRNA of IFN-γ, TNF-α, TNF-β, IFN-α, and IL2-receptor-α, 36–48 h after *in vitro* stimulation (Mwangi et al. 1998, 2002). This cytokine profile is typical of cell-mediated immune responses to intracellular infection and highlights the role of γδ-T lymphocytes in immunity to pathogens. IFN-γ plays an important role in protective immunity against *E. ruminantium* (Totte et al. 1993; Mahan et al. 1996) and intracellular organisms in general. It mediates its effect by direct killing of infected cells, by rendering host cells less "infection friendly," and by activating other cytokine pathways which kill intracellular organisms, in particular the activation of the phagocytes to release TNF-α and activate the nitric oxide pathway. However, excessive production of these IFN-γ induced downstream molecules may lead to tissue destruction and pathology. The role of $CD8^+$ T cells remains unclear in ruminants, but in mice they appear to contribute to the pathology of the infection.

E. ruminantium evades the host's immune response by several methods. First, it can survive inside phagocytic cells and seems to inhibit fusion with destructive lysosomes. Although there is no direct evidence for this observation for *E. ruminantium*, this phenomenon is recognized for other ehrlichial pathogens that reside inside macrophages (Wells and Rikihisa 1988; Kumagai et al. 2006). Second, *E. ruminantium* causes a downregulation of MHC class I and II antigen expression on endothelial cells *in vitro* (Vachiery et al. 1998) which may suppress antigen presentation via the MHC class I and II pathways and inhibit induction of $CD8^+$ and $CD4^+$ T cell immunity. Third, *E. ruminantium* uses the phenomenon of antigenic variation to survive in its host and prevent elimination, which is discussed below.

The desired method of control of *E. ruminantium* infection is by the development of safe efficacious

vaccines that activate the TH-1 immune response pathway. Two vaccines have been developed to date. A vaccine containing inactivated *E. ruminantium* in combination with an adjuvant that stimulates cell-mediated immunity protects sheep, goats, and cattle against lethal challenge (Martinez et al. 1996; Totte et al. 1997; Mahan et al. 1998, 2001; Vachiery et al. 2006). This vaccine induces the TH-1 immune pathway (reviewed in Mahan et al. 2003). DNA or genetic vaccines have also been tested against *E. ruminantium* infection. The MAP-1 DNA vaccine activates the Th-1 pathway (secretion of high levels of IFN-γ) and protects inbred DBA/2 mice against *E. ruminantium* challenge. However, vaccination with the recombinant MAP-1 protein without DNA vaccine priming induces nonprotective Th-2 responses, further endorsing the fact that protective immunity against *E. ruminantium* is cell mediated (Nyika et al. 2002). Several other protective genes of *E. ruminantium* have been identified and their evaluation in mice and sheep is being pursued. An attenuated vaccine based on cell culture-derived organisms is also being explored (Zweygarth et al. 2005, 2008).

Genes Associated with Pathogenic Mechanisms of **Ehrlichia ruminantium**

The circular genome of *E. ruminantium* is represented on a single chromosome. The predicted genome size from the sequences of *E. ruminantium* Welgevonden and Gardel strains is 1.5 megabases, consisting of 920–950 coding sequences (Collins et al. 2005; Frutos et al. 2006). The *E ruminantium* genome has marked synteny with the genome of *E. canis*, *E. chaffeensis*, and *Anaplasma* spp. (Hotopp et al. 2006; Mavromatis et al. 2006). This genome depicts reductive evolution of an aerobic organism and contains genes representing the central metabolic pathway without the glycolytic pathway genes; genes involved in energy metabolism, information transfer, membrane transport systems, and pathogenicity; numerous membrane protein genes and a high representation of tandemly repeated and duplicated sequences (some typical of ankyrin repeats); and pseudogenes (mostly truncated) and three multigene families.

Adhesion to and invasion of host cells are two mechanisms that facilitate pathogen entry and are usually accomplished by protein molecules anchored on the pathogen's surface encoded by adhesin and invasin genes. One adhesin gene of *E. ruminantium* has been described. This gene called "mucin" encodes a protein of 35.7 kDa and facilitates adhesion to tick cells *in vitro*. It has nine tandemly repeated amino acid sequences, which is typical of genes involved in similar functions in related pathogens (de la Fuente et al. 2004). Adhesion genes responsible for interaction with eukaryotic host cells have not been described so far. Invasin genes of *E. ruminantium* have also not been described, but there are numerous genes in its genome that encode putative unique OMPs.

There are a few genes that encode for molecules which allow the pathogen to escape hostile host responses. All Rickettsiales contain the *sodB* gene (superoxide dismutase) which is responsible for neutralizing the reactive oxygen-mediated defense mechanisms of the hosts. Virulence genes (*vir*) are present as operons (*VirB3-11* and *VirD4*) which are considered to be associated with pathogenicity and represent the type IV secretion system. This system transports proteins and DNA of pathogen origin into the host cell and is recognized in gram-negative intracellular bacteria. Vir proteins play a key role in host–pathogen interaction and inhibit lysosomal destruction by inhibiting trafficking to lysosomes. In *A. marginale*, certain gene products of the type IV secretion system are being proposed as vaccine candidates due to highly conserved antigenic sequences and because protected cattle make a strong $CD4^+$ T cell response reminiscent of protective immunity (Lopez et al. 2007).

Although *E. ruminantium* is a gram-negative bacterium, it lacks enzymes required for the biosynthesis of peptidoglycan or LPS. The absence of these molecules is probably an evolutionary mechanism to evade the host's immune response as the bacterium lacks the ligands that bind TLR-2 and TLR-4 on host antigen-presenting cells.

The high representation of sequence repeats in the genome (8.3% of the chromosome) signifies that this mechanism is important to *E. ruminantium*'s persistence. These repeats give rise to pseudogenes which are used to produce different allelic proteins and generate antigenic diversity, thereby evading the immune response.

There are three unique multigene families (or operons) of 16, 14, and 10 paralogs which are predicted to encode OMPs and to have a role in interaction with the host environment. While the biological function of these multigene families is not fully

known, the 16 paralog *map-1* gene family is located on a 25-kb locus and has been characterized due to the immunodominance of its encoded proteins. The *map-1* gene family shares high sequence homology with gene families of *E. canis* and *E. chaffeensis*. The *map1* gene encodes a conserved MAP-1 immunodominant surface protein of approximately 28 kDa which has been a focus of diagnostics and vaccine development. It is recognized by antisera raised against diverse *E. ruminantium* field isolates and by T cells of immune cattle, sheep, and goats. *map1*-DNA vaccines with or without MAP-1 recombinant protein boosts protection against homologous challenge in a mouse model; however, the hypervariability of this gene contributes to a lack of cross-protection from heterologous strains (Nyika et al. 2002). The members of this gene family have variable sequence identity as well as differential expression patterns. In endothelial cell cultures transcripts of all *map-1* paralogs are detected (van Heerden et al. 2004), whereas in tick cells and in *A. variegatum* ticks only *map-1* and *map-1-1* transcripts are detected (Bekker et al. 2002; Postigo et al. 2007). Transcripts of *map-1* and *map-1-1* genes were detected in midgut and salivary gland tissues of feeding ticks, and only the former was detected in midguts of unfed ticks, which depicts a biological role of this gene family during transmission of the pathogen. The *in vivo* significance of some of the gene family members needs full examination, especially because *in vitro*, the *map1* paralogs appear to be complete, functional, and generate mRNA transcripts. The *map1* gene and the upstream-located *map1-2* gene have the highest level of sequence diversity between *E. ruminantium* strains of 84–100% and 75–100%, respectively. Interestingly, strains can have similar *map1* but different *map1-2*'s. The *map1-2*'s vary independently of *map-1*, and different *map1-2*'s were found in sheep (Barbet et al. 2009). Antisera from some field-infected cattle recognize MAP-1-2B variable region peptide but less consistently and strongly than the MAP-1B peptide of MAP-1. One prediction about the *map1* and *map1-2* gene diversity is that different regions of the genes are subject to more or less variation due to the evolutionary pressures. The sequence variation of this gene family and of the genome sequence at large is predicted to be a result of recombination events in the tick and homologous recombination events which have impacted the evolution of host–pathogen interactions and evasion of the host's immune response (Hughes and French 2007). These findings suggest that heterologous protective immunity in heartwater and related ehrlichia may be dependent on recognition of a complex set of sequence varying peptides which justifies further testing the *map1* paralogs in animals to determine their respective protective roles. In addition, this sequence diversity ensures the persistence of the organism in the infected host.

Ehrlichia muris

Background, Etiology, and Epidemiology

In 1983, an infectious agent designated as strain AS145 was isolated from the spleen of a wild mouse captured during a routine survey in Japan (Kawahara et al. 1993). When a spleen homogenate was inoculated intraperitoneally into BALB/c mice, the mice developed clinical signs of lethargy, anorexia, and ruffled fur about 10 days post inoculation. At necropsy, splenomegaly was present. A spleen homogenate was then inoculated into seven strains of 8-week-old mice. At about day 10 post inoculation, the mice developed ruffled fur, inactivity, and anorexia. The maximum spleen weight was about 10 times the normal value. The mortality rate was less than 1.0%. Among the strains of mice tested, BALB/c showed the greatest degree of splenomegaly.

Compact and round inclusion bodies were seen in the cytoplasm of peritoneal macrophages of infected mice. Electron microscopy suggested that the organism was ultrastructurally similar to those of the genus *Ehrlichia*. Serological cross-reactivities were tested among a variety of ehrlichial strains. The AS145 isolate reacted only with the *E. canis* group. The mouse serum against strain AS145 reacted in Western blots with 64-, 47-, 46-, 44-, and 40-kDa proteins of *E. canis* and the 64-kDa protein of *Ehrlichia risticii, Ehrlichia sennetsu*, and *Neorickettsia helminthoeca*. Amplification of the 16S rRNA revealed a similarity of 97.9% to *E. chaffeensis*. This was consistent with the results of an immunoblot analysis performed with immune sera against various ehrlichiosis agents and on the basis of these findings and other morphological, biological, and serological characteristics, the organism was assigned to a new species, *Ehrlichia muris* (Wen et al. 1995b). The tick *Haemophysalis flava* may be the potential vector of *E. muris* (Kawahara et al. 1999). *H. flava* has only been recognized in Japan and Korea. A serological survey revealed that

exposure to *E. muris* or to organisms antigenically cross-reactive with *E. muris* occurred among dogs, wild mice, monkeys, bears, deer, and wild boars. The seropositivity rate in humans is low, with low antibody titers. Clinical signs of seropositive humans are unknown but are considered to be absent or mild (Kawahara et al. 1999). The geographic distribution, animal reservoirs, vectors, and the rate of human exposure are unknown.

Pathogensis

A Th-1-type response is the predominant immune reaction to *E. muris* infection as indicated by increased blood TNF-γ levels, which peaked at day 10 post infection (Kawahara et al. 1996). Infected mice developed a marked hypergammaglobulinemia of IgM and IgG. Of the IgG types, IgG2a and IgG3 increased, while IgG1 and IgG2b remained constant. Despite the hypergammaglobulinemia, both IgG and IgM antibody titers against *E. muris* were very low throughout the 30-day study period, probably due to an impairment of antigen-specific immune stimulation. In spite of the hypergammaglobulinemia and increased blood TNF-α levels, clearance of the organism was incomplete and the mice remained persistent carriers for up to 400 days post infection.

A further mechanism for antibody production has been demonstrated by studying $CD4^+$ T cell deficient C57BL/6 mice. In these mice, a population of antigen-specific plasmablasts has been identified in the extrafollicular region of the spleen which is responsible for T-independent antibody production (Racine et al. 2008).

Because of the close genetic and antigenic relationships among *E. chaffeensis*, *E. canis*, and *E. muris*, the latter animal model is considered advantageous for elucidation of the pathogenesis, protective immune mechanisms, and the evaluation of vaccines against monocytotrophic ehrlichiae (Feng and Walker 2004). Although *E. muris* does not cause a fatal infection in wild-type mice, a variety of KO mice were used to determine which factors are important in immunity to acute monocytotrophic *E. muris* infection. Exceptionally, C57BL/6 mice were found to possess an inherent strong resistance to *E. chaffeensis* and *E. muris* infection despite the use of a range of KO mice. The nature of this resistance remains undefined. In other strains of mice, the importance of $CD8^+$ and $CD4^+$ T lymphocytes, antibodies, and IFN-γ combined with TNF-α has been demonstrated in the protective immunity of mice against *E. muris* infection (Feng and Walker 2004).

E. muris inoculated intradermally into C57BL/6 mice protected them from challenge by the highly virulent mouse strain of *Ehrlichia* from *Ixodes ovatus* (IOE) (Stevenson et al. 2006). The authors proposed that this protection could be due to two interrelated factors: an initial regional containment of the bacteria along with the promotion of an accelerated priming of *Ehrlichia*-specific T cells secondary to optimal and effective antigen presenting cells, leading to the generation of a strong protective type-1 response.

E. muris infection in immunocompetent mice (AKR and C57BL/6 strains) has been proposed as an animal model for persistent subclinical ehrlichiosis. The histopathologic progress of the acute *E. muris* infection and the subsequent subclinical infection revealed similar patterns to those of infections caused by *E. chaffeenis* (Dumler et al. 1993; Walker and Dumler 1997). Infection of mononuclear phagocytic cells was followed by the development of lesions in the liver, lungs, spleen, and bone marrow similar to those described in humans and other animal models. Mild histological lesions were still present in some organs 150 days after infection, and low levels of ehrlichial DNA could be detected in organs examined by real-time PCR. The presence of persistently high titers of antibodies in the sera of infected mice was suggestive of a continuous stimulation of the immune system by *E. muris* (Kawahara et al. 1996, 1999). Furthermore, the pattern of immunoreactive proteins detected by Western blot from AKR and C57BL/6 mice infected by *E. muris* appeared to correspond to the major immunoreactive proteins identified in *E. canis* infection (McBride et al. 2003). Cellular immunity is generally considered the dominant form of protection against intracellular bacterial infections, via $CD4^+$ T cells and their production of type 1 cytokines (IFN-γ, TNF-α, IL-12). However, type 1 cytokines were not detected in mice challenged with the highly pathogenic IOE strain after induction of immunity by *E. muris*. Antisera from both wild-type and MHC-II-deficient mice provided partial resistance to IOE challenge, and protection was also achieved by transfer of splenocytes from *E. muris*-immunized mice (Bitsaktsis et al. 2007).

Neorickettsia helminthoeca and *Neorickettsia risticii*

Background, Etiology, and Epidemiology

Neorickettsia helminthoeca is the cause of salmon poisoning disease, a highly fatal disease of dogs in the Pacific Northwest United States. The disease is transmitted by *Nanophyetus salmincola*, a small intestinal trematode of dogs (Gorham and Foreyt 2006). Its life cycle involves the dog, a freshwater snail, and a salmonid fish. Coyotes and foxes also show clinical signs, but cats do not. The incubation period is about a week and signs include fever, anorexia, lymph node enlargement, and severe hemorrhagic enteritis which may be fatal in 7–10 days if untreated. Diagnosis is based on the presence of intracellular pleomorphic rods that fill the cytoplasm of the mononuclear phagocytic cells in lymph node aspirates. Finding ova of *N. salmincola* in the animal's feces supports the diagnosis. There is no vaccine against the neorickettsiae, and infections are best prevented by avoiding consumption of infected salmonid fish.

Equine monocytic neorickettsiosis (Potomac horse fever) is an acute enterocolitis of horses in North America that is caused by *Neorickettsia risticii* and closely related species (Wen et al. 1995a). The vectors are digenetic trematodes that use freshwater snails and aquatic insects, such as caddisflies, as intermediate hosts. It is not known how infections are transmitted from the aquatic insects to horses but insectivores, such as bats and swallows, may be natural reservoirs (Pusterla et al. 2003). *N. risticii* circulates in monocytes and has a predilection for the mucosa of the cecum and large colon. Most infections are subclinical and when signs appear they are very variable, including combinations of fever, depression, anorexia, colic, and ileus. Diarrhea occurs in under 60% of horses and laminitis in up to 40% of cases (Palmer 2004). The mortality rate can reach 30%; signs in horses that survive without treatment usually resolve over 5–10 days. Organisms are seldom seen in blood smears, serology is unreliable, and diagnosis is best based on PCR detection. Inactivated, partially purified, whole cell vaccines are available but protection is short lived (around 4 months) and vaccine failures occur, possibly because of lack of cross-protection among strains of *N. risticii*.

Dogs experimentally infected with *N. risticii* show no clinical signs (Ristic et al. 1988), but naturally acquired infections have been described with signs of fever, bleeding tendencies, edema, neurological signs, polyarthritis, anemia, and thrombocytopenia. It is not certain whether the agent is *N. risticii* or a caninotropic strain of the organism (Kakoma et al. 1994).

Pathophysiology of Neorickettsioses

Most of the data on the pathophysiology of neorickettsiae are derived from studies on *N. risticii*. After ingestion, metacercariae develop into adult trematodes which attach to the intestinal mucosa and inoculate neorickettsiae by an unknown mechanism. Following initial replication, which probably takes place in epithelial cells of the villi or intestinal lymphoid tissue (Gorham and Foreyt 2006), organisms enter the blood early in the course of the disease and spread to the lymph nodes, spleen, tonsils, thymus, lungs, and brain. There is marked enlargement of the lymph nodes, which show a marked depletion in mature lymphocytes and foci of necrosis in the cortex and medulla (Rikihisa et al. 1985). Neorickettsiae do not generally stimulate severe inflammatory reactions in tissues (Rikihisa et al. 1985), probably because of lack of complete macrophage activation. Infected cells do not secrete IL-1 or TNF, although they kill organisms when activated by exogenous stimuli that raise intracellular calcium levels (Rikihisa 1991).

Neorickettsiae are pleomorphic coccoid organisms that are nonmotile and enter host cells (monocytes, macrophages, mast cells, and intestinal epithelial cells) by receptor-mediated endocytosis. The host cell receptors and neorickettsial ligands appear to be proteins (Messick and Rikihisa 1993), and the endocytosis requires transglutaminase activity and possibly receptor-coated pits in the host membrane (Rikihisa et al. 1995). It is, however, independent of microfilaments which are associated with phagocytosis. Monocytes and macrophages have no intrinsic resistance to infection, while intestinal epithelial cells, which are not normally phagocytic, are induced to take up the organisms (Rikihisa 1991). Internalization and subsequent proliferation and spread within macrophages depend on increased cytosolic free calcium and calmodulin (Rikihisa et al. 1995). Within cells, the neorickettsiae tend to aggregate on one side of the cytoplasm. Each organism is tightly encased by host cell membrane and this cytoplasmic endosome does not fuse with lysosomes in the macrophages of their mammalian

hosts. Lysosomal fusion is only inhibited with parasitophorous vacuoles; fusion still occurs with other uninfected vacuoles, even within the same cell (Wells and Rikihisa 1988). Neorickettsiae that enter neutrophils are destroyed (Messick and Rikihisa 1993).

Movement within cells relies on calcium-dependent assembly of cytoskeletal microfilaments and microtubules (Rikihisa et al. 1995). Transmission between cells occurs following release of organisms from the host cell by exocytosis or cytolysis, which usually occurs only after cells become filled completely by infecting organisms (Rikihisa 1991). Adjacent cells take up organisms by endocytosis with fusion of the inclusion membrane to that of the host cell.

Growth of neorickettsiae is dependent on iron which is obtained from the labile pool in the host cytoplasm (Rikihisa et al. 1994). Following infection there is up-regulation of TfR mRNA in the host cell as a result of activation of iron regulating factors (e.g., iron responsive protein [IRP]) which prevent degradation of the mRNA (Barnewall et al. 1999). Neorickettsiae lack a conventional glycolytic pathway and the electron transport chain with its iron-containing cytochromes may be the sole mechanism of ATP generation. The organisms use glutamine rather than glutamate to generate ATP as it penetrates the endosome more effectively. This process appears to be dependent on free calcium within the organisms but is not directly dependent on calmodulin or functional calcium channels in the cell membranes (Rikihisa et al. 1995).

In macrophages, and probably also in intestinal epithelial cells, organisms multiply by binary fission. Division occurs in early endosomes enriched with cytoplasmic TfR (Barnewall et al. 1999).

Humoral and cell-mediated immune responses are important in protection. The Fab fragments of antibodies to neorickettsiae prevent them from binding to macrophages and prevent receptor-mediated endocytosis (Messick and Rikihisa 1994). Organisms coated with antineorickettsia IgG, however, readily enter cells via the Fc receptor, although they are unable to replicate and survive in the cells. This suggests that IgG prevents infections of intestinal cells which lack Fc receptor. The reactive IgG probably kills neorickettsiae by inhibiting their energy metabolism, which leads to lysosomes fusing with inclusion membranes. It is not clear if the reduced metabolism induced by antibodies is due to inhibition of L-glutamine transport or a specific enzyme or due to steric hindrance of the energy transduction process (Messick and Rikihisa 1994).

Neorickettsiae appear to cause immunosuppression, with spleen and blood antigen-specific lymphocyte responses being decreased (Rikihisa et al. 1987; Rikihisa 1991). Inhibition of antigen-specific T lymphocyte activation is suggested by the suppression of class II histocompatibility antigen (Ia antigen) induction on the surface of infected macrophages in response to IFN-γ (Rikihisa 1991).

FAMILY RICKETTSIACEAE

Rickettsia rickettsii, *Rickettsia conorii*, and *Rickettsia felis*

Background, Etiology, and Epidemiology

Rickettsia rickettsii is the agent of Rocky Mountain spotted fever (RMSF) in dogs and humans (Greene and Breitschwerdt 2006). The organism occurs in Central and South America but is most widely reported in North America where it is transmitted principally by *D. andersoni* and *D. variabilis*. Most infections in dogs are subclinical, and high percentages of apparently healthy dogs are seropositive in endemic areas. Other nonpathogenic SFG rickettsiae, including *Rickettsia rhipicephali*, *Rickettsia montana* and *Rickettsia belli*, may stimulate immune responses in dogs which protect them from infections with *Rickettsia rickettsii* (Breitschwerdt et al. 1988c).

Dogs with clinical signs often have fever, depression, anorexia, lymph node enlargement, subcutaneous edema, muscle and joint pain, and petechiation of the skin and mucous membranes. There might also be epistaxis, melena, hematuria, focal neurological signs, necrosis of the extremities, and death from hemorrhagic diathesis, failure of vital organs, or vascular collapse and shock. *Rickettsia conorii* is transmitted by the brown dog tick, *Rhipicephalus sanguineus*, and is the agent of Mediterranean spotted fever in people around the Mediterranean basin and in Africa and Asia (Parola et al. 2005). Clinical signs in humans are similar to those of RMSF but the disease is far milder and the mortality rate low. Although *Rickettsia conorii* has been found in the blood of dogs suffering from an acute febrile illness (Solano-Gallego et al. 2006), experimentally infected dogs showed no clinical or laboratory abnormalities. They did, however, seroconvert

and were rickettsemic for up to 10 days (Kelly et al. 1992).

Rickettsia felis is the recently described agent of flea-borne spotted fever in people. The organism is maintained in nature by the cat flea, *Ctenocephalides felis*. Cats show no clinical signs when infected but they become rickettsemic for short periods (less than a month) before reactive antibodies develop and clear the infection (Wedincamp and Foil 2000).

There is little information on the effects of the numerous other SFG rickettsiae on domestic animals. In limited studies, SFG rickettsiae that are nonpathogenic in people have also been found not to cause disease in animals. There are no commercial vaccines, and prevention depends on tick control.

Pathogenesis of SFG Rickettsial Infections

Most data on the pathophysiology of SFG rickettsial infections are derived from studies on *Rickettsia rickettsii* and *Rickettsia conorii* infections of people and laboratory animals. Ticks containing virulent rickettsiae in their salivary glands inject the organisms into the host and, at the site of the bite, the rickettsiae localize in contiguous endothelial cells, the most important target cells in the body (Walker et al. 1982). Adherence to the target endothelial cells is a critical step in the establishment of successful infection (Martinez and Cossart 2004). The rickettsiae attach to host cell receptor Ku70 (Martinez et al. 2005), a subunit of DNA-dependent protein kinase, by means of adhesins such as rickettsial OMP A (rOmpA) and rickettsial OMP B (rOmpB). The latter recruits additional Ku70 molecules, and ubiquitin ligase (c-Cbl) is also recruited to the entry foci. Ubiquination of Ku70 is followed by activation of the actin-related protein 2/3 complex (Arp2/3) by multiple pathways involving Cdc42 (a small guanidine triphosphatase), phosphoinositide 3-kinase (PI 3-kinase), tyrosine kinase (c-Src), focal adhesion kinase (FAK), and cortactin (Martinez and Cossart 2004). The resultant actin polymerization changes the actin cytoskeletal structure at entry sites, and the host cell membrane wraps or “zippers” around the organisms with phagocytosis of organisms into the cell.

The resultant phagosome lyses very rapidly, probably by release of phospholipase D and hemolysin C from the rickettsiae which disrupts the phagosomal membrane (Walker 2007). The release of organisms enables them to escape exposure to lysosomal enzymes and enter the nutrient-rich cytoplasmic environment which has enabled rickettsiae to jettison genes encoding for enzymes, sugar metabolism, lipid, nucleotide, and amino acid synthesis (Walker 2007). The rickettsiae are thus obligate intracellular organisms. They divide by binary fission within the cell, and there is no extracellular replication.

Intercellular spread of SFG rickettsiae occurs by actin-based motility (ABM), as is the case with *Listeria*, *Shigella*, and vaccinia virus (Gouin et al. 1999; Van Kirk et al. 2000; Heinzen 2003). How polymerization of host cell actin is directed by rickettsiae is controversial (Balraj et al. 2008), but it results in the formation of a typical F-actin “comet tail.” These are usually over 10-μm long and consist of multiple, distinct actin bundles wrapped around one another in a helical fashion. Formation of “comet tails” propels the rickettsiae across the host cell cytoplasm. When organisms encounter a stationary object in the cytoplasm, an actin tail can form on the opposite pole of the organism enabling multidirectional movement. The ABM can also push the rickettsiae against the host cell membrane to form protrusions which are engulfed by neighboring cells and enable spread of infection without exposure to host antibodies. The mechanism of actin tail formation in rickettsiae is unknown, but it differs from previously described ABM systems (Heinzen 2003). Cytoskeletal proteins probably involved in the formation of the tails include vasodilator-stimulated phosphoprotein, profiling, vinculin, and filamin. Ezrin, paxillin, and tropomyosin proteins involved in ABM of other organisms are not found in rickettsiae (Van Kirk et al. 2000).

Some organisms escape from infected cells and spread via the lymphatics to regional lymph nodes, causing lymphadenomegaly. They also enter the blood stream and infect endothelial cells throughout the body, but particularly in the lungs, brain, liver, kidneys, heart, spleen, and skin. Damage to the endothelial cells results in necrotizing vasculitis with infiltration of T cells and macrophages, the histopathologic hallmark of rickettsial disease (Keenan et al. 1977; Toutous-Trellu et al. 2003). This vasculitis leads to increased vascular permeability, edema, hyponatremia, and elevated aldosterone and antidiuretic hormone (ADH) levels (Greene and Breitschwerdt 2006). Hypotension may occur, and there might be inappropriate ADH release. In fulminating infections, endothelial damage might be

severe and result in vascular collapse and death. Following infection, apoptosis of endothelial cells is delayed and this enables continued growth of rickettsiae and prolonged intracellular survival of the organisms. The delay results from activation of nuclear factor $_{\kappa}$B, which maintains mitochondrial integrity of host cells and protects against infection-induced apoptosis by preventing activation of caspase-9- and caspase-8-mediated pathways (Joshi et al. 2003).

Electron microscopic changes seen in endothelial cells as a result of infection include elongation to a spindle shape, development of interendothelial gaps, formation of stress fibers, and disruption of cell junctions (Valbuena and Walker 2005). These changes probably contribute to increased vascular permeability and may enable infected endothelial cells to enter the circulation and spread rickettsiae around the body (Walker et al. 2003). Changes in the permeability of the endothelial monolayer are due to the intracellular rickettsiae themselves and also to the immune responses generated against the organisms (Woods and Olano 2007). Increased permeability is directly related to the number of rickettsiae and is associated with a decrease in transendothelial electrical resistance, an effect which is markedly enhanced by proinflammatory stimuli essential to rickettsial immunity. The increased permeability is not dependent on the presence of nitric oxide and is associated with dissociation of adherence junctions. Infection of endothelial cells results in altered expression of adhesion molecules, regulatory cytokines, and the antioxidant enzyme heme oxygenase.

A recent study has shown that infection induces the endothelial cyclooxygenase-2 (COX-2) system, and the resultant enhanced release of vasoactive prostaglandins may contribute to the vascular permeability changes during SFG rickettsioses and also to the regulation of inflammatory responses (Rydkina et al. 2006). Infection also results in a rapid and dose dependent activation of the p38 subfamily of mitogen-activated protein (MAP) kinases which are important signaling pathways that coordinate intracellular responses to infections. Rickettsiae might exploit the activation of MAP kinase signaling to facilitate their own internalization and replication (Rydkina et al. 2005) and inhibiting p38 MAP kinase has been suggested as a possible therapeutic strategy against vasculitis during rickettsial infections.

Infection of endothelia can also result in cell death due to direct injury to host cell membranes by free radicals. ROS produced by infected endothelial cells damage host cell membranes by lipid peroxidation and also deplete host defenses against ROS such as glucose-6-phosphate dehydrogenase, glutathione peroxidise, and catalase (Walker et al. 1984; Hong et al. 1998). There is some evidence that rickettsial phospholipase D and/or protease activity may also be involved in cell injury (Walker et al. 1984; Silverman 1997; Renesto et al. 2003; Walker 2007), but there is no indication that secreted toxins are involved. The role of cytokines, other mediators of inflammation, and host immune mechanisms in endothelial cell death are yet to be determined (Walker 2007).

Damage to endothelial cells results in platelet activation and decreasing platelet numbers (Davidson et al. 1990; Elghetany and Walker 1999). Thrombocytopenia might be exacerbated by autoimmune reactions as platelet-associated immunoglobulin levels are elevated (Grindem et al. 1999). Infections result in a hypercoagulable state as endothelial injury results in release of procoagulant components, activation of the coagulation cascade with thrombin generation, platelet activation, increased antifibrinolytic factors, consumption of natural anticoagulants, and possibly high levels of coagulation-promoting cytokines (Elghetany and Walker 1999). Although antithrombin III and plasminogen levels decrease and fibrinogen degradation products increase, overt disseminated intravascular coagulation is uncommon (Walker 1989; Elghetany and Walker 1999).

Humoral immunity appears to play little role in controlling primary infections as reactive antibodies are usually not found until infections are controlled by other means. In subsequent exposures, however, humoral immunity appears to be important. Polyclonal antibodies and monoclonal antibodies to rOmpA and rOmpB provide passive immunity to virulent challenge (Feng et al. 2004a). Also, Fc-dependent polyclonal antibodies and monoclonal antibodies to rOmpB prevented organisms escaping from the phagosome (Feng et al. 2004b).

Cellular immunity is particularly important in primary exposure as rickettsiae are intracellular organisms (Walker et al. 2001). Infected endothelial cells produce proinflammatory cytokines including IL-6, IL-8, and monocyte chemoattractant protein (Jensenius et al. 2003; Walker 2007). T

lymphocytes and macrophages are attracted to sites of infection and surround vessels containing infected endothelial cells. Infected endothelial cells also express adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (Dignat-George et al. 1997; Vitale et al. 1999). The adhesion molecules enhance adherence of leukocytes to infected endothelial cells and mediate the rolling of the cells along the blood vessel wall and their migration to areas of inflammation in and below the endothelium. This focusing of effector cells is an essential component of the inflammatory response during rickettsial infection and may account for vascular injury (Dignat-George et al. 1997). On the other hand, selectin is also found free in the blood, and this might bind to leukocytes in circulation impeding their exudation, and thereby perhaps modulating the inflammatory response (Vitale et al. 1999).

Expression of various other chemokines that target T lymphocytes (e.g., CXCL9 and CXCL10) is also increased with infection, but these appear not to be chemoattractants for effector T cells (Valbuena et al. 2003). It has been hypothesized that they may play a role in guiding effector cells of the innate immune system such as macrophages and natural killer cells.

T lymphocytes and macrophages at infection sites appear to produce IFN-γ and TNF-α. These cytokines are thought to activate endothelial cells to kill rickettsiae. Their clearance from cells is associated with the appearance of fragments of organisms in the cytoplasm which may form large aggregates. Rickettsiae are killed by mechanisms in the endothelial cells and macrophages involving ROS such as superoxide, hydroxyl radicals, inducible nitric oxide synthase (iNOS), and hydrogen peroxide (Walker et al. 1997). How ROS can be both a host defense and a pathogenic mechanism is not clear, but it has been suggested that different subcellular compartments are involved (Walker et al. 2003). Starving rickettsiae of tryptophan by antibody-induced indoleamine-2,3-dioxygenase (IDO), which degrades the amino acid, also plays a role in the killing of the organisms. A recent study has shown mild to moderate SFG rickettsial infections are associated with a strong and balanced intralesional proinflammatory and anti-inflammatory response (elevated TNF-α, IFN-γ, iNOS, and IDO) and a dominant type 1 immunity. Severe Mediterranean spotted fever is associated with increased expression of chemokine mRNA (regulated upon activation, normally T-cell expressed, and secreted [RANTES]) (de Sousa et al. 2007).

Rickettsiae are also killed when infected endothelial cells and macrophages are targeted by cytoxic MHC-I-restricted T lymphocytes. The cytotoxic T lymphocyte (CTL) activity of these recruited $CD8^{+}$ T lymphocytes is more critical in the clearance of rickettsiae than is the production of IFN-γ (Walker et al. 2001). The CTLs induce apoptosis of infected cells, the remnants of which are phagocytosed by surrounding macrophages and the rickettsiae they contain destroyed in phagolysosomes.

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